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#### CORRECTIONS

On page 166, Vol. xcv, No. 1, February, 1932, line 7 from the foot of the page, read *0.19* for *0.27*.

On page 174, line 10 from the top, read (3) for (2).

On page 178, Table VI, column 10, sixth figure, read *5.92* for *3.02*; last figure, read *3.85* for *1.95*.



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## CONFIGURATIONAL RELATIONSHIP OF HYDRO-CARBONS

### IV. OPTICAL ROTATIONS OF HYDROCARBONS OF THE ISOAMYL SERIES. THE CONFIGURATIONAL RELATIONSHIP OF SUBSTITUTED CARBONIC ACIDS CONTAINING AN ISOBUTYL AND AN ISOAMYL GROUP TO THOSE OF THE CORRESPONDING NORMAL CARBONIC ACIDS

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(Received for publication, November 16, 1931)

The present communication deals with optically active hydrocarbons of the homologous series of 1,1-methylisoamylpropanes in which the methyl and isoamyl groups remain unchanged.

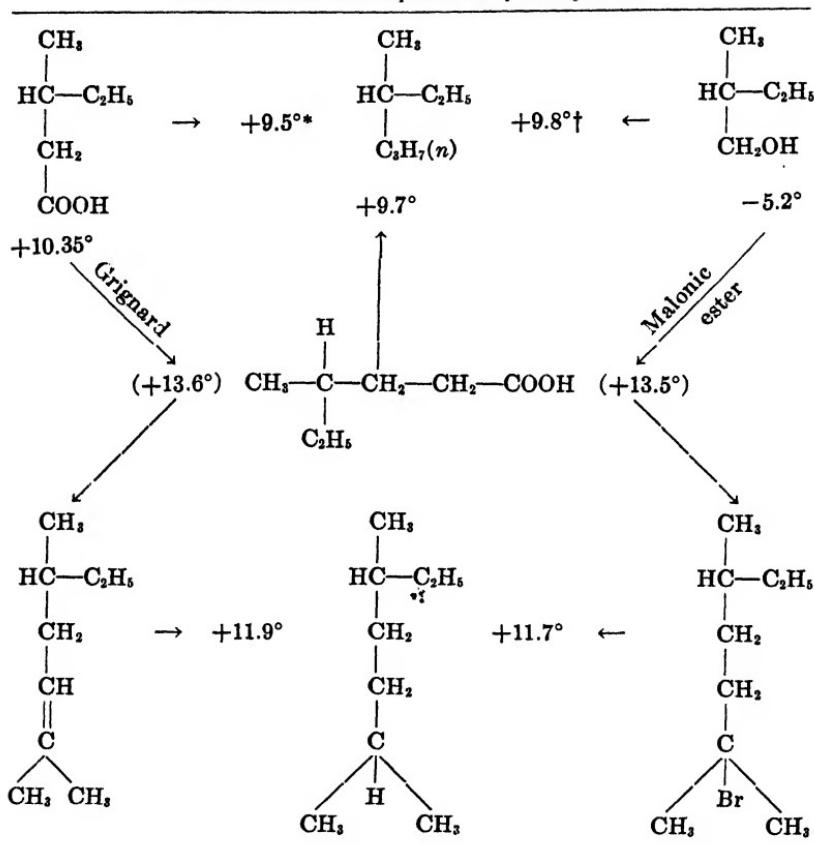
In Papers I and III<sup>1</sup> of this series it was shown that the distance of the isopropyl group from the asymmetric carbon atom was an important factor determining the direction and value of the rotation of the hydrocarbon. Thus, optically active trisubstituted methanes containing an isopropyl group rotated in the opposite direction from those containing an isobutyl group in place of the isopropyl. The lower members of the isobutyl series rotated in the same direction as the members of the normal series, their values of rotation being higher in the members of the isobutyl series. The members beyond the butylisobutyl member rotated in the opposite direction from the normal, their values being lower.

As regards the isoamyl series, two possibilities were to be considered. If distance alone played the major part in determining the effect of the isopropyl group, then the members of this series should rotate in the same direction as the members of the isobutyl series; were the even or the odd position with respect to the asymmetric carbon atom the determining factor, then the members

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931); **92**, 455 (1931).

of the isoamyl series might be expected to rotate in the same direction, as those of the isopropyl series, or at least might have a rotation value lower than that of the members of the normal

TABLE I  
Maximum Molecular Rotations from Experimental Data to Show No  
Racemization in Preparation of Compounds



\* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 82 (1931).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 764 (1931).

series. Indeed, the methylethylisoamylmethane previously prepared by us had a value of rotation lower than that of the corresponding member of the normal series.<sup>2</sup> However, when the

<sup>2</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931).

higher members of the isoamyl series were prepared, it was observed that the values of their rotations approached very closely those of the members of the normal series. It was therefore considered necessary to reinvestigate the value of the rotation of the methylethylisoamylmethane. A scrutiny of every step in the preparation of the hydrocarbon aroused the suspicion that one of these steps might have been accompanied by considerable racemization; namely, the dehydration of the 2-ethyl-5-dimethylpentanol-5 which had been accomplished by means of iodine at the boiling temperature of the carbinol. The dehydration was therefore repeated with oxalic acid as a dehydrating agent. Under these conditions, the reaction was accomplished at a lower temperature, thus reducing the danger of racemization. The methylethylisoamylmethane prepared in this manner had a higher rotation; namely,  $[M]_D^{25} = +11.9^\circ$ , in place of  $+7.09^\circ$  as previously reported. In order to test the correctness of this value, the hydrocarbon was also prepared by reduction of 1-bromo-1,1-dimethyl-4-ethylpentane by shaking with palladium and hydrogen. A practically identical value was obtained for the rotation of the hydrocarbon; namely,  $[M]_D^{25} = +11.7^\circ$ . *Thus we are confident that the value of the molecular rotation of the methylethylisoamylmethane as now obtained is correct (Table I).*

*Preparation and Configurational Relationships of Higher Members of Methylethylisoamylmethane Series*—The starting materials for the higher members of this series were the configurationally related butanoic acids-4 substituted in position (2). The acids of this series have been correlated by the direct chemical method and therefore the hydrocarbons derived from them may be correlated among themselves on a reliable basis. The individual steps for the preparation of the hydrocarbons are shown in Table II. In Table II are given the configurationally related substituted butanoic acids-4 and the hydrocarbons derived from them. It may be seen that the configurationally related hydrocarbons containing the isoamyl group including the methylamylisoamylmethane rotate in the same direction. The values of rotation given in Table II are experimental, and not maximum rotations.

*Extrapolation of Maximum Rotations*—The maximum rotations were calculated by multiplying the observed values by the ratio maximum rotation / observed rotation of the parent substance. This mode of calcula-

# Hydrocarbon Configurations. IV

tion is permissible in cases where racemization is excluded. The test in the case of methylethylisoamylmethane described above justifies the assumption that the reactions leading from the butanoic acids-4 substituted in position (2) are not accompanied

TABLE II  
*Experimental Values Obtained in Preparation of Hydrocarbons Containing a Methyl and an Isoamyl Group.  $[M]_D^{**}$*

	$-\text{CH}_2\text{-COOH}$	$-\text{CH}_2\text{-CH}_2\text{Br}$	$-\text{CH}_2\text{-CH}_2\text{-COOH}$	$-\text{CH}_2\text{-CH}_2\text{-COOC}_2\text{H}_5$	$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2\text{-CH}=\text{C} \\   \\ \text{CH}_3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2\text{-CH}_2\text{-CH} \\   \\ \text{CH}_3 \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_2\text{H}_5 \end{array}$	-3.04	-6.51*	-4.02	-3.76	-3.01	-3.52
$\begin{array}{c} \text{CH}_3\ddagger \\   \\ \text{HC}- \\   \\ \text{C}_3\text{H}_7(n) \end{array}$	+1.59	-9.29	-3.04	-2.43	-1.83	-1.55
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_4\text{H}_9(n) \end{array}$	+3.14	-9.01	-2.10	-1.17	-0.91	-0.76
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_5\text{H}_{11}(n) \end{array}$	+4.56	-8.30	-1.01	-0.24	-0.59	-0.04

\* The chloride in this case was used instead of the bromide.

† For convenience of discussion, the signs of all members of this series were changed from those found experimentally.

by racemization. Even had racemization occurred, it should have been of the same order of magnitude in the entire series of derivatives so that the differences in the rotations of members of homologous series should be of approximately the same order of magnitude as in the case in which racemization had not occurred.

Maximum Molecular Rotations of Configurationally Related Hydrocarbons (in Homogeneous Solution) of the General Type  $H - \overset{\text{CH}_3}{\underset{\text{R}_1}{\text{C}}} - R_2$   
 Where  $R_1$  and  $R_2$  are Alkyl Radicals.  $[M]_D^{\text{obs}}$

(1)	(2)	(3)	(4)	(5)	(6) -	(7)	(8)
$\text{CH}_3$ HCOH $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $+10.3$	$\text{CH}_3$ $\text{HC} - \text{CH}_2 - \text{COOH}$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $-10.35$	$\text{CH}_3$ $\text{HC} - \text{C}_2\text{H}_5$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $0$	$\text{CH}_3$ $\text{HC} - \text{C}_3\text{H}_7(n)$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $-9.9$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(n)$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $-11.4$	$\text{CH}_3$ $\text{HC} - \text{C}_5\text{H}_{11}(n)$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $-12.0$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(\text{iso})$ $\overset{\text{C}_2\text{H}_5}{\text{C}_2\text{H}_5}$ $-21.3$	$\text{CH}_3$ $\text{HC} - \text{C}_6\text{H}_{11}(\text{iso})$ $\overset{\text{C}_2\text{H}_6}{\text{C}_2\text{H}_6}$ $-11.9$
$\text{CH}_3$ HCOH $\overset{\text{C}_2\text{H}_7(n)}{\text{C}_2\text{H}_7}$ $+12.2$	$\text{CH}_3$ $\text{HC} - \text{CH}_2 - \text{COOH}$ $\overset{\text{C}_2\text{H}_7(n)}{\text{C}_2\text{H}_7}$ $+3.60$	$\text{CH}_3$ $\text{HC} - \text{C}_2\text{H}_5$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $+9.9$	$\text{CH}_3$ $\text{HC} - \text{C}_3\text{H}_7(n)$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $0$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(n)$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $-1.7$	$\text{CH}_3$ $\text{HC} - \text{C}_5\text{H}_{11}(n)$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $-2.4$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(\text{iso})$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $-14.9$	$\text{CH}_3$ $\text{HC} - \text{C}_6\text{H}_{11}(\text{iso})$ $\overset{\text{C}_3\text{H}_7(n)}{\text{C}_3\text{H}_7}$ $-3.5$
$\text{CH}_3$ HCOH $\overset{\text{C}_2\text{H}_7(n)}{\text{C}_2\text{H}_7}$ $+11.8$	$\text{CH}_3$ $\text{HC} - \text{CH}_2 - \text{COOH}$ $\overset{\text{C}_2\text{H}_7(n)}{\text{C}_2\text{H}_7}$ $+6.06$	$\text{CH}_3$ $\text{HC} - \text{C}_2\text{H}_5$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $+11.4$	$\text{CH}_3$ $\text{HC} - \text{C}_3\text{H}_7(n)$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $+1.5$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(n)$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $0$	$\text{CH}_3$ $\text{HC} - \text{C}_5\text{H}_{11}(n)$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $-0.8$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(\text{iso})$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $-11.9$	$\text{CH}_3$ $\text{HC} - \text{C}_6\text{H}_{11}(\text{iso})$ $\overset{\text{C}_4\text{H}_9(n)}{\text{C}_4\text{H}_9}$ $-1.5$
$\text{CH}_3$ HCOH $\overset{\text{C}_2\text{H}_{11}(n)}{\text{C}_2\text{H}_{11}}$ $+11.8$	$\text{CH}_3$ $\text{HC} - \text{CH}_2 - \text{COOH}$ $\overset{\text{C}_2\text{H}_{11}(n)}{\text{C}_2\text{H}_{11}}$ $+8.12$	$\text{CH}_3$ $\text{HC} - \text{C}_2\text{H}_5$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $+12.5$	$\text{CH}_3$ $\text{HC} - \text{C}_3\text{H}_7(n)$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $+2.4$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(n)$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $+0.8$	$\text{CH}_3$ $\text{HC} - \text{C}_5\text{H}_{11}(n)$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $0$	$\text{CH}_3$ $\text{HC} - \text{C}_4\text{H}_9(\text{iso})$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $-9.3$	$\text{CH}_3$ $\text{HC} - \text{C}_6\text{H}_{11}(\text{iso})$ $\overset{\text{C}_3\text{H}_{11}(n)}{\text{C}_3\text{H}_{11}}$ $-0.2$

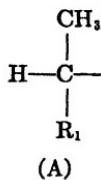
\* These values are calculated on the basis of maximum rotations of the corresponding propionic acids from which they were prepared (Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **92**, 456 (1931)).

The values of rotation of the members of the normal, isobutyl, and isoamyl series are given in Table III.

#### CONCLUSIONS

From Table III it may be seen that the members of the isoamyl series of hydrocarbons have approximately the same value of rotation as the members of the normal amyl series. Thus, it would seem that in the case of aliphatic hydrocarbons, *the isopropyl group introduces a special rotatory contribution only when located either directly on the asymmetric carbon atom or at a distance of 1 carbon only from the asymmetric carbon atom.*

A second important conclusion may be formulated from a scrutiny of the data contained in Table III. If one compares a series of homologous hydrocarbons with a corresponding series of carbinols, as, for instance, the one given in Column 1 of Table III, the following is observed: The values of the rotations in the series of carbinols progressively increase. The values of the rotations of the hydrocarbons of Column 3 likewise progressively increase and have the same sign as those of the carbinols. The values in Column 4 decrease from the first to the third member and then progressively increase, the sign having changed after the symmetric member. In Columns 5 and 6, the values of all the members above the symmetric progressively decline, the sign being opposite from that of the carbinols, whereas those of the members below the symmetric progressively increase, having the same sign as the corresponding carbinols. The values of the members of the isobutyl and of the isopropyl series progressively decline in value. All these changes in values may be explained on the assumption that the rotation of every one of these substances is composed of two major contributions of opposite sign. In a simplified way, to the radicle



the positive value A may be assigned and to the group R<sub>2</sub> the negative contribution (B). Thus, when A is greater than B, the

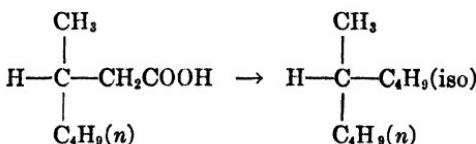
substance rotates in the direction of A, and, as the value of A increases, the value of the rotation of the substance increases. On the other hand, when the value of B is greater than that of A, then the direction of rotation of the substance is negative, and, if the value of B remains constant, A now increasing, then the values of the rotation of the substances will progressively decline. On the other hand, if the value A remains constant, the value B increasing, then the values of the substances will decline until the symmetric member is reached and will increase beyond that member. Such a condition is observed in the horizontal rows of Table III.

Returning now to the isobutyl and isoamyl series, it is evident that, in the light of the above discussion, *the isobutyl group has a higher negative value than the normal butyl, whereas the isoamyl group has only a slightly higher value than the corresponding normal group.*

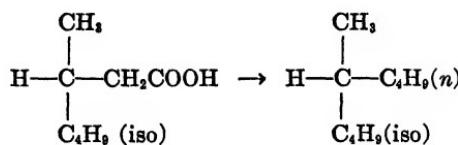
We wish to emphasize, however, that in speaking of the contribution of any one group we do not wish to attribute to it an independent value, but a value which is itself the result of the vicinal effect of all the other groups. This applies equally to groups A and B. We also wish to emphasize that the value of group B in every vertical column remains only approximately constant.

*Configurational Relationship of Substituted Carbonic Acids Containing an Isobutyl and an Isoamyl Group to Those of Corresponding Carbonic Acids Containing a Normal Butyl or a Normal Amyl Group*

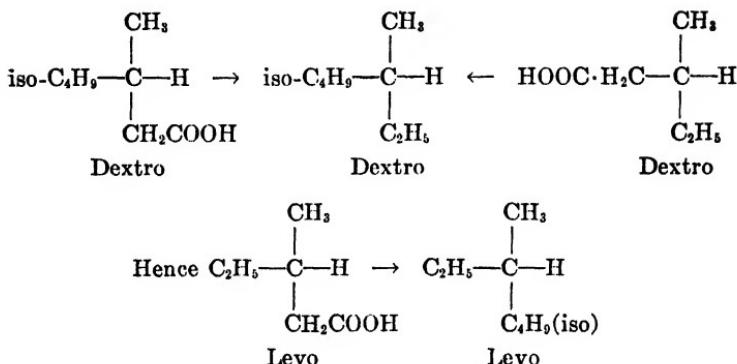
Since the configurations of the hydrocarbons with respect to the disubstituted carbonic acids have been established, it has now become possible to correlate the configurations of the above two types of carbonic acids. From the following formulae it may be seen that the two acids which lead to enantiomorphous hydrocar-



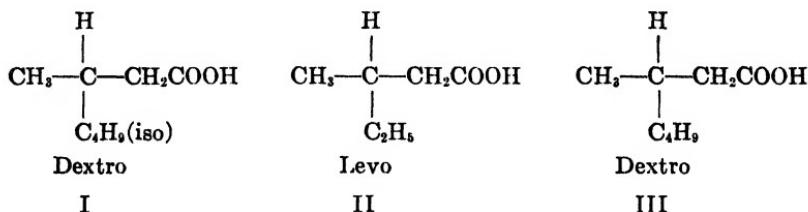
## Hydrocarbon Configurations. IV



bons are configurationally related. The following were the reactions actually performed.

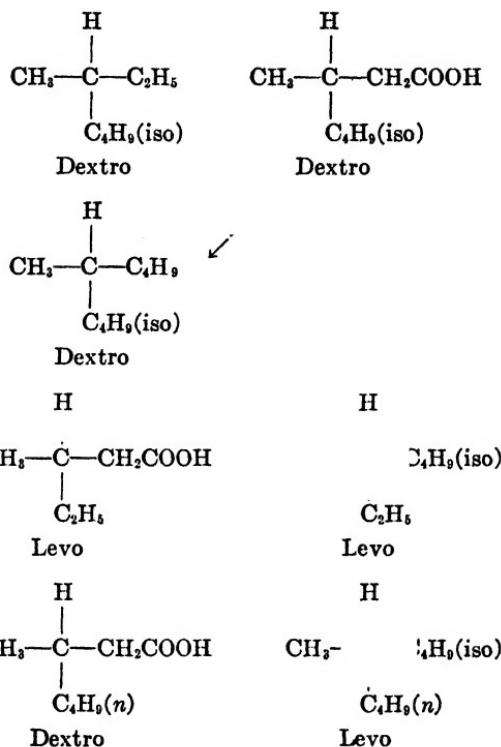


*From this it follows that dextro-2-isobutylbutanoic acid-4 is configurationally related to levo-2-ethylbutanoic acid-4. This means that the following two acids may be regarded as configurationally related, inasmuch as in projection the isobutyl and the ethyl groups are located on the same side of the butanoic acid when they rotate in opposite directions.*



Furthermore, since levo-2-ethylbutanoic acid-4 is configurationally related to dextro-2-butylbutanoic acid-4, it follows that 2-isobutylbutanoic acid-4 and 2-butylbutanoic acid-4 are configurationally related when they rotate in the same direction. *It is also evident that 2-butylbutanoic acid-4 and 2-isobutylbutanoic acid-4 rotating in*

*the same direction will lead to hydrocarbons rotating in opposite directions.*



The configurational relationship of the 2-isoamylbutanoic acid-4 to the corresponding normal acid was established in the same way, inasmuch as dextro-2-ethylbutanoic acid-4 and dextro-2-isoamylbutanoic acid-4 both lead to dextro-methylethylisoamylmethane.

*Maximum Rotations of 2-Isobutyl- and of 2-Isoamylbutanoic Acids-4*—The original task in the preparation of these two acids was their resolution to the maximum rotations with the object of preparing the hydrocarbons of the isobutyl and of the isoamyl series by methods which would definitely exclude the possibility of racemization. Unfortunately, the resolution of these two acids proved a very difficult task. However, the knowledge of the so called maximum rotations of the hydrocarbons of the isobutyl and of the isoamyl series makes it possible to calculate a value for the

rotations of 2-isobutyl and of 2-isoamylbutanoic acids-4. These values then permit of an estimate of the effect on the rotation of substituting the butyl group by an isobutyl and the amyl by an isoamyl. Thus, multiplying the experimental value for the molecular rotation of 2-isobutylbutanoic acid-4 by the ratio  $\frac{21.7}{3.4}$

TABLE IV  
*Comparison of Configurational Relationship of Isobutyl and Isoamyl Derivatives to n-Butyl and n-Amyl Derivatives*  
 Experimental results (not maximum rotations).  $[M]_D^{25}$ .

	-CH <sub>2</sub> -COOH	-CH <sub>2</sub> -COOC <sub>2</sub> H <sub>5</sub>	-CH <sub>2</sub> -CH <sub>2</sub> OH	-CH <sub>2</sub> -CH <sub>2</sub> Br	-CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>2</sub> OH	-CH <sub>2</sub> -	-C <sub>2</sub> H <sub>5</sub>	-C <sub>3</sub> H <sub>7</sub> (n)
CH <sub>3</sub> *	+5.5	+2.6	+3.6	-15.0	+0.7	+10.3		+1.4
HC- C <sub>4</sub> H <sub>9</sub> (n)								
CH <sub>3</sub>	+2.4	+1.9	+1.9	-1.0			+3.4	
HC- C <sub>4</sub> H <sub>9</sub> (iso)								
CH <sub>3</sub> *	+7.1	+3.7	+5.4	-13.0	+2.3	+11.0	+2.1	
HC- C <sub>5</sub> H <sub>11</sub> (n)								
CH <sub>3</sub>	+3.8	+2.6	+3.2	-4.6	+1.3	+5.4	+1.4	
HC- C <sub>5</sub> H <sub>11</sub> (iso)								

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

value of 15.0° for the rotation of the acid is obtained, whereas the corresponding normal acid has a rotation of 6.0°. The 2-isoamylbutanoic acid-4, on the same principle, has a maximum molecular rotation of  $\frac{3.8 \times 11.9}{-5.4} = 8.40^\circ$  against a value of 8.1°

for the corresponding normal acid. Thus, the substitution of a normal butyl group by an isobutyl group enhances the rotation considerably. The substitution of a normal amyl group by an isoamyl group has little effect on the rotation (see Table IV).

#### EXPERIMENTAL

*Dextro-2-Ethylvaleric Acid-5*—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 40 gm. of 1-chloro-3-methylpentane,  $[M]_D^{25} = +6.51^\circ$  (from 2-ethylbutyric acid-4,  $[M]_D^{25} = +3.04^\circ$ ). The solution was cooled and dry carbon dioxide passed into it for about 30 minutes. The Grignard solution was decomposed in the usual way, and the ether distilled. The acid was purified through its sodium salt. This was then decomposed and the organic acid distilled. B.p.  $115^\circ$  at 16 mm. Yield 29 gm.

$$[\alpha]_D^{22} = \frac{+2.85^\circ}{1 \times 0.923} = +3.09^\circ; [M]_D^{22} = +4.02^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{22} = +13.59^\circ$  (homogeneous).

The maximum rotation calculated from the 2-ethylbutyric acid-4 was in agreement with that previously obtained from 1-bromo-2-methylbutane and malonic ester.<sup>3</sup>

2. 848 mg. substance: 6.700 mg. CO<sub>2</sub> and 2.838 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>14</sub>O<sub>2</sub>. Calculated. C 64.6, H 10.8  
 Found. " 64.2, " 11.2

*Dextro-Ethyl Ester of 2-Ethylvaleric Acid-5*—59 gm. of 2-ethylvaleric acid-5,  $[M]_D^{22} = +4.02^\circ$ , were dissolved in 150 cc. of absolute alcohol, and 5 cc. of concentrated sulfuric acid were added. The product was refluxed on a steam bath for  $\frac{1}{2}$  hour, the excess alcohol distilled off under reduced pressure, water added, and the ester extracted with ether. It was then distilled. B.p.  $80^\circ$  at 20 mm. Yield 60 gm. D  $\frac{22}{4} = 0.888$ .

$$[\alpha]_D^{22} = \frac{+2.11^\circ}{1 \times 0.888} = +2.38^\circ; [M]_D^{22} = +3.76^\circ \text{ (homogeneous)}$$

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<sup>3</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 79 (1931).

3.277 mg. substance: 8.185 mg. CO<sub>2</sub> and 3.389 mg. H<sub>2</sub>O  
 C<sub>9</sub>H<sub>16</sub>O. Calculated. C 68.3, H 11.5  
 Found. " 68.1, " 11.6

*Dextro-4-Methyl-1-Hexanol*—50 gm. of ethyl ester of 3-methylcaproic acid-6, [M]<sub>D</sub><sup>25</sup> = +2.60°, were dissolved in 200 cc. of absolute alcohol and then reduced by dropping into a suspension of 90 gm. of sodium in 450 cc. of toluene with rapid stirring. The carbinol was isolated and purified as previously described. B.p. 77° at 20 mm. Yield 31 gm. D  $\frac{28}{4} = 0.818$ .

$$[\alpha]_D^{28} = \frac{+1.79^\circ}{1 \times 0.818} = +2.19^\circ; [M]_D^{28} = +2.54^\circ \text{ (homogeneous)}$$

Calculated maximum [M]<sub>D</sub><sup>28</sup> = +11.9° (homogeneous).

3.930 mg. substance: 10.430 mg. CO<sub>2</sub> and 4.930 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>16</sub>O. Calculated. C 72.3, H 13.9  
 Found. " 72.1, " 14.0

*Dextro-4-Methyl-1-Bromohexane*—100 gm. of phosphorus tribromide were slowly added to 62 gm. of 4-methyl-1-hexanol, [M]<sub>D</sub><sup>28</sup> = +2.54°. The product was heated on a steam bath for 15 minutes, poured onto ice, and the halide extracted with ether. The ether was evaporated and the bromide purified in the usual way. B.p. 78° at 44 mm. Yield 65 gm.

$$[\alpha]_D^{28} = \frac{+2.95^\circ}{1 \times 1.129} = +2.61^\circ; [M]_D^{28} = +4.67^\circ \text{ (homogeneous)}$$

Calculated maximum [M]<sub>D</sub><sup>28</sup> = +21.9° (homogeneous).

5.835 mg. substance: 10.060 mg. CO<sub>2</sub> and 4.520 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>16</sub>Br. Calculated. C 46.9, H 8.5  
 Found. " 47.0, " 8.6

*Dextro-Methylethylpropylmethane*—A Grignard reagent was prepared from 25 gm. of 4-methyl-1-bromohexane, [M]<sub>D</sub><sup>28</sup> = +2.44°, and 3 gm. of magnesium in 50 cc. of dry ether. The ethereal solution was poured onto ice and the hydrocarbon extracted with ether. The ether was distilled. The hydrocarbon was shaken with cold concentrated sulfuric acid, then with sodium carbonate, and

finally washed with water. It was dried with sodium sulfate and then distilled from sodium. B.p. 92° at 760 mm. Yield 4 gm.

$$D \frac{28}{4} = 0.681.$$

$$[\alpha]_D^{28} = \frac{+1.12^\circ}{1 \times 0.681} = +1.64^\circ; [M]_D^{28} = +1.65^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{28} = +9.67^\circ$  (homogeneous).

4.714 mg. substance: 14.555 mg. CO<sub>2</sub> and 6.686 mg. H<sub>2</sub>O  
 $C_7H_{16}$ . Calculated. C 83.9, H 16.1  
 Found. " 84.2, " 15.9

*Dextro-Methylethylisoamylmethane*—60 gm. of ethyl ester of 2-ethylvaleric acid-5,  $[M]_D^{22} = +3.76^\circ$ , were added to 1 mol of methylmagnesium iodide in dry ether. The Grignard solution was poured onto ice and ammonium chloride solution and the carbinol was extracted with ether. The ether solution was dried with anhydrous sodium sulfate; then the ether was distilled off under reduced pressure. The carbinol was not distilled.

30 gm. of crude carbinol were mixed with 10 gm. of oxalic acid and slowly distilled at atmospheric pressure. The operation was repeated. The distillate was separated from the water, dried with sodium sulfate, and the unsaturated hydrocarbon distilled. B.p. 138° at 760 mm. Yield 17 gm.

$$[\alpha]_D^{28} = \frac{+1.77^\circ}{1 \times 0.740} = +2.39^\circ; [M]_D^{28} = +3.02^\circ \text{ (homogeneous)}$$

(a) To 10 gm. of the unsaturated hydrocarbon was added 1 gm. of platinic oxide. Reduction was carried out by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction was complete in 15 minutes. The hydrocarbon was shaken with cold, concentrated sulfuric acid, washed with water and sodium carbonate solution, dried with sodium sulfate, then distilled from a small piece of sodium. B.p. 134° at 760 mm.

$$\text{Yield } 8 \text{ gm. } D \frac{22}{4} = 0.717.$$

$$[\alpha]_D^{28} = \frac{+1.97^\circ}{1 \times 0.717} = +2.75^\circ; [M]_D^{28} = +3.52^\circ \text{ (homogeneous)}$$

4.376 mg. substance: 13.561 mg. CO<sub>2</sub> and 6.105 mg. H<sub>2</sub>O  
 C<sub>8</sub>H<sub>20</sub>. Calculated. C 84.3, H 15.7  
 Found. " 84.5, " 15.6

(b) 20 gm. of the carbinol were shaken with 200 cc. of cold hydrobromic acid. This was warmed on a steam bath for  $\frac{1}{2}$  hour. The oil which separated was distilled. B.p. 92° at 20 mm. Yield 24 gm. This was placed in a flask with 100 cc. of 10 per cent sodium hydroxide and 1 gm. of colloidal palladium. It was shaken with hydrogen for 24 hours. The hydrocarbon was extracted with ether and distilled. It was purified as previously described, then redistilled from sodium. B.p. 134° at 760 mm. Yield 5 gm.

$$[\alpha]_D^u = \frac{+1.95^\circ}{1 \times 0.717} = +2.72^\circ; [M]_D^u = +3.48^\circ \text{ (homogeneous)}$$

3.999 mg. substance: 12.352 mg. CO<sub>2</sub> and 5.610 mg. H<sub>2</sub>O  
 C<sub>8</sub>H<sub>20</sub>. Calculated. C 84.3, H 15.7  
 Found. " 84.2, " 15.7

*Dextro-2-Propylvaleric Acid-5*—A Grignard reagent was prepared from 6 gm. of magnesium in 100 cc. of dry ether and 45 gm. of 1-bromo-3-methylhexane, [M]<sub>D</sub><sup>21</sup> = +9.29° (from 2-propylbutyric acid-4, [α]<sub>D</sub><sup>25</sup> = -1.22°). Dry carbon dioxide was passed for  $\frac{1}{2}$  hour into the cooled Grignard solution. The product was poured onto ice and hydrochloric acid, and the organic acid extracted with ether. It was then distilled. B.p. 132° at 22 mm.

Yield 31 gm. D  $\frac{24}{4} = 0.882$ .

$$[\alpha]_D^u = \frac{+1.86^\circ}{1 \times 0.882} = +2.11^\circ; [M]_D^u = +3.04^\circ \text{ (homogeneous)}$$

3.475 mg. substance: 8.525 mg. CO<sub>2</sub> and 3.500 mg. H<sub>2</sub>O  
 C<sub>8</sub>H<sub>16</sub>O<sub>2</sub>. Calculated. C 66.6, H 11.2  
 Found. " 66.9, " 11.3

*Dextro-Ethyl Ester of 2-Propylvaleric Acid-5*—31 gm. of 2-propylvaleric acid-5, [M]<sub>D</sub><sup>25</sup> = +3.04°, were dissolved in 100 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. This was refluxed  $\frac{1}{2}$  hour on a steam bath. The excess alcohol was distilled off under reduced pressure, water was added, and the

ester extracted with ether. The solution was dried with sodium sulfate, then distilled. B.p. 104° at 32 mm. Yield 33 gm.

$$D \frac{24}{4} = 0.859.$$

$$[\alpha]_D^{24} = +1.21^\circ \quad 1 \times 0.859 \quad +1.41^\circ; [M]_D^{24} = +2.43^\circ \text{ (homogeneous)}$$

3.685 mg. substance: 9.505 mg. CO<sub>2</sub> and 4.000 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>. Calculated. C 69.7, H 11.7

Found. " 70.3, " 12.1

*Dextro-Methyl-n-Propylisoamylmethane*—33 gm. of ethyl ester of 2-propylvaleric acid-5, [M]<sub>D</sub><sup>24</sup> = +2.43°, were added to 0.5 mol of methylmagnesium iodide in dry ether. The Grignard solution was decomposed by ice and ammonium chloride solution and the carbinol extracted with ether. The ether was distilled and the residue distilled from 12 gm. of oxalic acid. The unsaturated hydrocarbon was separated from the water, dried, and distilled from sodium. B.p. 162°. Yield 14 gm.

$$[\alpha]_D^{24} = +0.98^\circ \quad 1 \times 0.750 = +1.31^\circ; [M]_D^{24} = +1.83^\circ \text{ (homogeneous)}$$

To 14 gm. of the unsaturated hydrocarbon was added 1 gm. of platinic oxide. The hydrocarbon was reduced by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction was complete in 15 minutes. The hydrocarbon was shaken with cold concentrated sulfuric acid, then washed with water and sodium carbonate solution, dried with sodium sulfate, and distilled from sodium. B.p. 156° at 760 mm. Yield 12 gm.

$$D \frac{25}{4} = 0.725.$$

$$[\alpha]_D^{25} = +0.79^\circ \quad 1 \times 0.725 = +1.09^\circ; [\alpha]_D^{25} = +1.55^\circ \text{ (homogeneous)}$$

4.412 mg. substance: 13.675 mg. CO<sub>2</sub> and 6.200 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>. Calculated. C 84.4, H 15.6

Found. " 84.5, " 15.7

*Levo-2-n-Butylvaleric Acid-5*—A Grignard reagent was prepared from 6 gm. of magnesium in 100 cc. of dry ether and 50 gm. of 1-bromo-3-methylheptane, [M]<sub>D</sub><sup>24</sup> = -9.01° (from 2-butylbutyric

acid-4,  $[\alpha]_D^{23} = +2.18^\circ$ ). This was cooled in ice and carbon dioxide passed in for  $\frac{1}{2}$  hour. The Grignard solution was poured onto ice and hydrochloric acid. The organic acid was extracted with ether, the ether evaporated, and the acid purified by extracting an aqueous solution of its sodium salt with ether. The acid was then distilled. B.p.  $149^\circ$  at 22 mm. Yield 33 gm.  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-1.17^\circ}{1 \times 0.871} = -1.34^\circ; [M]_D^{25} = -2.13^\circ \text{ (homogeneous)}$$

3.191 mg. substance: 7.935 mg.  $\text{CO}_2$  and 3.320 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{9}\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.5  
 Found. " 67.8, " 11.6

*Levo-Ethyl Ester of 2-n-Butylvaleric Acid-5*—33 gm. of 2-n-butylvaleric acid-5,  $[M]_D^{25} = -2.10^\circ$ , were mixed with 100 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid added. The product was refluxed on a steam bath for  $\frac{1}{2}$  hour, then the excess alcohol was distilled off under reduced pressure. Water was added and the ester extracted with ether. It was then distilled.

B.p.  $112^\circ$  at 23 mm. Yield 36 gm.  $D \frac{25}{4} = 0.861$ .

$$[\alpha]_D^{25} = \frac{-0.55^\circ}{1 \times 0.861} = -0.64^\circ; [M]_D^{25} = -1.19^\circ \text{ (homogeneous)}$$

2.779 mg. substance: 7.200 mg.  $\text{CO}_2$  and 3.055 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{11}\text{H}_{22}\text{O}_2$ . Calculated. C 70.9, H 11.9  
 Found. " 70.7, " 12.3

*Levo-Methyl-n-Butylisoamylmethane*—To 1 mol of methylmagnesium iodide in ether were added 36 gm. of ethyl ester of 2-n-butylvaleric acid-5,  $[M]_D^{25} = -1.17^\circ$ . The Grignard solution was poured onto ice and ammonium chloride, and the carbinol was extracted with ether. The ether was evaporated under reduced pressure and the residue heated at  $150^\circ$  for  $\frac{1}{2}$  hour with 20 gm. of oxalic acid. The unsaturated hydrocarbon was then distilled under reduced pressure and finally from sodium. B.p.  $113^\circ$  at 100 mm. Yield 9 gm.

$$[\alpha]_D^{25} = \frac{-0.44^\circ}{1 \times 0.759} = -0.58^\circ; [M]_D^{25} = -0.89^\circ \text{ (homogeneous)}$$

9 gm. of the unsaturated hydrocarbon were placed in a bottle with 1 gm. of platinic oxide and reduced by shaking with hydrogen under 30 pounds per square inch pressure. The hydrocarbon was purified as described for methylethylisoamylmethane. B.p. 109° at 100 mm. Yield 7 gm.  $D \frac{25}{4} = 0.738$ .

$$[\alpha]_D^{25} = \frac{-0.36^\circ}{1 \times 0.738} = -0.49^\circ; [M]_D^{25} = -0.76^\circ \text{ (homogeneous)}$$

4.341 mg. substance: 13.420 mg. CO<sub>2</sub> and 5.970 mg. H<sub>2</sub>O  
 $C_{11}H_{24}$ . Calculated. C 84.5, H 15.5  
 Found. " 84.3, " 15.4

*Levo-n-Amylvaleric Acid-5*—A Grignard reagent was prepared from 52 gm. of 1-bromo-3-methyloctane,  $[M]_D^{24} = -8.30^\circ$  (from 2-amylbutyric acid-4,  $[\alpha]_D^{25} = +2.92^\circ$ ). Carbon dioxide was passed for  $\frac{1}{2}$  hour into the Grignard solution cooled in ice. The reaction product was poured onto ice and hydrochloric acid, and the organic acid was extracted with ether. The ether was distilled off and the sodium salt of the acid was purified by extracting its aqueous solution with ether. The acid was then distilled.

B.p. 156° at 22 mm. Yield 37 gm.  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-0.52^\circ}{1 \times 0.871} = -0.60^\circ; [M]_D^{25} = -1.03^\circ \text{ (homogeneous)}$$

3.975 mg. substance: 10.135 mg. CO<sub>2</sub> and 4.145 mg. H<sub>2</sub>O  
 $C_{10}H_{20}O_2$ . Calculated. C 69.7, H 11.7  
 Found. " 69.5, " 11.7

*Levo-Ethyl Ester of 2-n-Amylvaleric Acid-5*—37 gm. of 2-n-amylvaleric acid-5,  $[M]_D^{25} = -1.01^\circ$ , were added to 100 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated  $\frac{1}{2}$  hour on a steam bath, and then the excess alcohol was distilled off under reduced pressure. Water was added and the ester extracted with ether. It was then distilled. B.p.

120° at 22 mm. Yield 41 gm.  $D \frac{25}{4} = 0.862$ .

$$[\alpha]_D^{25} = \frac{-0.20^\circ}{2 \times 0.862} = -0.12^\circ; [M]_D^{25} = -0.23^\circ \text{ (homogeneous)}$$

2.452 mg. substance: 6.440 mg. CO<sub>2</sub> and 2.685 mg. H<sub>2</sub>O  
 C<sub>11</sub>H<sub>24</sub>O<sub>2</sub>. Calculated. C 71.9, H 12.1  
 Found. " 71.6, " 12.2

*Levo-Methyl-n-Amylisoamylmethane*—41 gm. of ethyl ester of 2-*n*-amylvaleric acid-5, [M]<sub>D</sub><sup>25</sup> = -0.24°, were added to 1 mol of methylmagnesium iodide in 300 cc. of dry ether. The Grignard solution was poured onto ice and ammonium chloride solution. The carbinol was extracted with ether and the ether distilled. The residue was heated at 150° for  $\frac{1}{2}$  hour with 25 gm. of oxalic acid, then distilled under reduced pressure. The unsaturated hydrocarbon was separated from the water, dried, and distilled from sodium. B.p. 125° at 100 mm. Yield 21 gm.

$$[\alpha]_D^{25} = \frac{-0.27^\circ}{1 \times 0.766} = -0.35^\circ; [M]_D^{25} = -0.59^\circ \text{ (homogeneous)}$$

14 gm. of the unsaturated hydrocarbon were placed in a flask with 1 gm. of platinic oxide and reduced by shaking with hydrogen under 30 pounds per square inch pressure. The hydrocarbon was purified as described for methylethylisoamylmethane. B.p. 122° at 100 mm. Yield 12 gm. D  $\frac{25}{4} = 0.739$ .

$$[\alpha]_D^{25} = \frac{-0.04^\circ}{1 \times 0.739} = -0.05^\circ; [M]_D^{25} = -0.09^\circ \text{ (homogeneous)}$$

4.986 mg. substance: 15.474 mg. CO<sub>2</sub> and 6.895 mg. H<sub>2</sub>O  
 C<sub>12</sub>H<sub>26</sub>. Calculated. C 84.6, H 15.4  
 Found. " 84.6, " 15.5

*Resolution of 2-*n*-Butylbutyric Acid-4*—This acid was resolved by crystallizing its quinine salt from acetone at -15° until the rotation of the acid reached a constant value. This was after the eighth crystallization.

$$[\alpha]_D^{25} = \frac{-3.81^\circ}{1 \times 0.905} = -4.21^\circ; [M]_D^{25} = -6.06^\circ \text{ (homogeneous)}$$

4.885 mg. substance: 11.945 mg. CO<sub>2</sub> and 4.950 mg. H<sub>2</sub>O  
 C<sub>8</sub>H<sub>16</sub>O<sub>2</sub>. Calculated. C 66.6, H 11.2  
 Found. " 66.7, " 11.3

*Resolution of 2-n-Amylbutyric Acid-4*—This acid was resolved by crystallizing its quinine salt from acetone at  $-15^{\circ}$  until the rotation of the free acid reached a constant value. This was reached on the eighth crystallization.

$$[\alpha]_D^n = \frac{-4.61^{\circ}}{1 \times 0.896} = -5.14^{\circ}; [M]_D^n = -8.12^{\circ} \text{ (homogeneous)}$$

5.964 mg. substance: 15.025 mg. CO<sub>2</sub> and 6.145 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 68.3, H 11.5

Found. " 68.7, " 11.5

*Levo-4-Methylnonane*—A Grignard reagent was prepared from 3 gm. of magnesium in 50 cc. of dry ether and 22 gm. of 1-bromo-4-methylnonane, [M]<sub>D</sub><sup>27</sup> = +5.57<sup>o</sup>.<sup>3</sup> The Grignard solution was decomposed with ice and hydrochloric acid. The hydrocarbon was extracted with ether and, after the usual purification, was distilled from sodium. B.p. 76° at 30 mm. Yield 5 gm. D  $\frac{27}{4}$  = 0.726.

$$[\alpha]_D^n = \frac{-1.13^{\circ}}{1 \times 0.726} = -1.56^{\circ}; [M]_D^n = -2.21^{\circ} \text{ (homogeneous)}$$

Calculated maximum [M]<sub>D</sub><sup>27</sup> = -2.47° (homogeneous).

2.450 mg. substance: 7.560 mg. CO<sub>2</sub> and 3.430 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>. Calculated. C 84.4, H 15.6

Found. " 84.1, " 15.7

*Dextro-2-Isobutylbutyric Acid-4*—The inactive acid was prepared from methylisobutylbromomethane and malonic ester.

1 mol of the inactive acid was dissolved in 400 cc. of boiling acetone and 1 mol of quinine was added. The solution was then filtered and 100 cc. of water added. The solution was allowed to stand in a refrigerator at  $-15^{\circ}$  until crystallization took place. The salt was filtered and recrystallized three times from 80 per cent acetone. It is very soluble and crystallizes with difficulty. For these experiments the mother liquors from the first crystallization were used. The acetone was evaporated and the quinine salt decomposed with hydrochloric acid. The organic

acid was extracted with ether, purified through its sodium salt, and then distilled. B.p. 124° at 20 mm. D  $\frac{30}{4} = 0.899$ .

$$[\alpha]_D^{20} = \frac{+1.48^\circ}{1 \times 0.899} = +1.65^\circ; [M]_D^{20} = +2.37^\circ \text{ (homogeneous)}$$

3.881 mg. substance: 9.433 mg. CO<sub>2</sub> and 4.000 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>16</sub>O<sub>2</sub>. Calculated. C 66.6, H 11.2

Found. " 66.3, " 11.5

*Dextro-Ethyl Ester of 2-Isobutylbutyric Acid-4*—100 gm. of 2-isobutylbutyric acid-4, [M]<sub>D</sub><sup>30</sup> = +2.37° (homogeneous), were dissolved in 200 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid added. The product was heated  $\frac{1}{2}$  hour on the steam bath, then the excess alcohol was distilled. Water was added and the ester extracted with ether. The ether extract was shaken with dilute sodium carbonate, then dried, and fractionated. B.p. 85° at 20 mm. Yield 105 gm. D  $\frac{30}{4} = 0.856$ .

$$[\alpha]_D^{20} = \frac{+0.97^\circ}{1 \times 0.856} = +1.13^\circ; [M]_D^{20} = +1.95^\circ \text{ (homogeneous)}$$

3.675 mg. substance: 9.410 mg. CO<sub>2</sub> and 3.890 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>. Calculated. C 69.7, H 11.7

Found. " 69.8, " 11.8

*Dextro-3,5-Dimethylhexanol-1*—50 gm. of ethyl ester of 2-isobutylbutyric acid-4, [M]<sub>D</sub><sup>30</sup> = +1.94° (homogeneous), were dissolved in 200 cc. of absolute alcohol and the solution slowly dropped into a suspension of 100 gm. of sodium in boiling toluene with vigorous stirring. The excess sodium was destroyed by additional absolute alcohol. The solution was poured onto ice and the carbinol extracted with ether, dried, and distilled. B.p. 105° at 45 mm.

Yield 30 gm. D  $\frac{30}{4} = 0.815$ .

$$[\alpha]_D^{20} = \frac{+1.19^\circ}{1 \times 0.815} = +1.46^\circ; [M]_D^{20} = +1.90^\circ \text{ (homogeneous)}$$

3.334 mg. substance: 8.934 mg. CO<sub>2</sub> and 4.225 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>16</sub>O. Calculated. C 73.8, H 13.9

Found. " 73.1, " 14.2

*Levo-1-Bromo-3,5-Dimethylhexane*—60 gm. of 3,5-dimethylhexanol-1,  $[M]_D^{30} = +1.90^\circ$  (homogeneous), were cooled in ice and 100 gm. of phosphorus tribromide slowly added. The product was heated on a steam bath for  $\frac{1}{2}$  hour, poured onto ice, and the halide extracted with ether. The ether was distilled off and the residue treated with cold sulfuric acid to remove any unchanged carbinol. It was separated, washed with dilute sodium carbonate solution, then water. It was dried and distilled. B.p.  $91^\circ$  at 45 mm.

Yield 60 gm.  $D \frac{30}{4} = 1.099.$

$$[\alpha]_D^{30} = \frac{-0.55^\circ}{1 \times 1.099} = -0.50^\circ; [M]_D^{30} = -0.97^\circ \text{ (homogeneous)}$$

5.632 mg. substance: 10.326 mg. CO<sub>2</sub> and 4.518 mg. H<sub>2</sub>O  
 $C_8H_{17}Br$ . Calculated. C 49.7, H 8.9  
 Found. " 50.0, " 9.0

*Dextro-3,5-Dimethylhexane*—A Grignard reagent was prepared from 30 gm. of 1-bromo-3,5-dimethylhexane,  $[M]_D^{30} = -0.97^\circ$  (homogeneous), and 3 gm. of magnesium in 100 cc. of dry ether. This was poured onto ice and hydrochloric acid. The hydrocarbon was extracted with ether, dried, and the ether distilled. The residue in the distilling flask was treated as previously described for the purification of hydrocarbons. B.p.  $111-112^\circ$  at 760 mm.

Yield 10 gm.  $D \frac{30}{4} = 0.696.$

$$[\alpha]_D^{30} = \frac{+2.08^\circ}{1 \times 0.696} = +2.99^\circ; [M]_D^{30} = +3.41^\circ \text{ (homogeneous)}$$

4.146 mg. substance: 12.817 mg. CO<sub>2</sub> and 5.847 mg. H<sub>2</sub>O  
 $C_8H_{18}$ . Calculated. C 84.1, H 15.9  
 Found. " 84.2, " 15.8

*Dextro-2-Isoamylbutyric Acid-4*—The inactive acid was prepared from methylisoamylbromomethane and ethyl malonate.

1 mol of the acid was dissolved in 500 cc. of hot acetone and 1 mol of quinine added. When the quinine had dissolved completely, 500 cc. of water were added and the solution cooled at  $-15^\circ$  until crystallization set in. The resolution was very difficult, owing to the solubility of the quinine salt. A better result was

obtained by making use of the filtrate from the first crystallization than by attempting the recrystallization of the levo form. The acetone was evaporated from the filtrate and the quinine salt decomposed with hydrochloric acid. The organic acid was extracted with ether, purified through its sodium salt, then distilled. B.p.

140° at 25 mm.  $D \frac{26}{4} = 0.901$ .

$$[\alpha]_D^{25} = \frac{+2.20^\circ}{1 \times 0.901} = +2.44^\circ; [M]_D^{25} = +3.86^\circ \text{ (homogeneous)}$$

4.637 mg. substance: 11.675 mg. CO<sub>2</sub> and 4.665 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 58.3, H 11.5

Found. " 68.6, " 11.3

*Dextro-Ethyl Ester of 2-Isoamylbutyric Acid-4*—To 60 gm. of 2-isoamylbutyric acid-4,  $[\alpha]_D^{20} = +2.44^\circ$ , were added 200 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid. The mixture was heated  $\frac{1}{2}$  hour on a steam bath, the excess alcohol distilled off, and the ester extracted with ether. The ester was then distilled. B.p. 112° at 30 mm. Yield 62 gm.  $D \frac{25}{4} = 0.862$ .

$$[\alpha]_D^{25} = \frac{+1.20^\circ}{1 \times 0.862} = +1.39^\circ; [M]_D^{25} = +2.59^\circ \text{ (homogeneous)}$$

3.705 mg. substance: 9.625 mg. CO<sub>2</sub> and 4.040 mg. H<sub>2</sub>O

C<sub>11</sub>H<sub>22</sub>O<sub>2</sub>. Calculated. C 70.9, H 11.9

Found. " 70.8, " 12.0

*Dextro-2,5-Dimethylheptanol-7*—62 gm. of ethyl ester of 2-isooamylbutyric acid-4,  $[\alpha]_D^{25} = +1.39^\circ$ , were dissolved in 200 cc. of absolute ethyl alcohol and reduced by slowly dropping into a suspension of 100 gm. of sodium in 500 cc. of boiling toluene with rapid stirring. The carbinol was isolated as previously described.

B.p. 102° at 18 mm. Yield 38 gm.  $D \frac{27}{4} = 0.823$ .

$$[\alpha]_D^{25} = \frac{+1.82^\circ}{1 \times 0.823} = +2.21^\circ; [M]_D^{25} = +3.18^\circ \text{ (homogeneous)}$$

3.461 mg. substance: 9.400 mg. CO<sub>2</sub> and 4.396 mg. H<sub>2</sub>O

C<sub>11</sub>H<sub>20</sub>O. Calculated. C 74.9, H 14.0

Found. " 74.1, " 14.2

*Levo-7-Bromo-2,5-Dimethylheptane*—38 gm. of 2,5-dimethylheptanol-7,  $[\alpha]_D^{27} = +2.21^\circ$ , were cooled in ice and 100 gm. of phosphorus tribromide were added. The mixture was heated on a steam bath for 1 hour, poured onto ice, and the halide extracted with ether. The ether was distilled and the halide, purified by the usual method, was distilled. B.p.  $108^\circ$  at 25 mm. Yield 42 gm. D  $\frac{27}{4} = 1.090$ .

$$[\alpha]_D^{27} = \frac{-2.40^\circ}{1 \times 1.090} = -2.20^\circ; [M]_D^{27} = -4.56^\circ \text{ (homogeneous)}$$

5.096 mg. substance: 9.766 mg. CO<sub>2</sub> and 4.165 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>11</sub>Br. Calculated. C 52.2, H 9.2

Found. " 52.3, " 9.1

*Dextro-2,5-Dimethylheptane*—A Grignard reagent was prepared from 3 gm. of magnesium in dry ether and 20 gm. of 7-bromo-2,5-dimethylheptane,  $[\alpha]_D^{27} = -2.20^\circ$ . This was poured into water and the hydrocarbon extracted with ether, purified, and then distilled from sodium. B.p.  $134^\circ$  at 760 mm. Yield 4 gm.

$$[\alpha]_D^{27} = \frac{+2.99^\circ}{1 \times 0.713} = +4.19^\circ; [M]_D^{27} = +5.37^\circ \text{ (homogeneous)}$$

2.682 mg. substance: 8.299 mg. CO<sub>2</sub> and 3.800 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>20</sub>. Calculated. C 84.3, H 15.7

Found. " 84.4, " 15.8

*Dextro-2,5-Dimethyloctanol-8*—A Grignard reagent was prepared from 7 gm. of magnesium in ether and 60 gm. of 7-bromo-2,5-dimethylheptane,  $[\alpha]_D^{27} = -2.20^\circ$ . To this were added 12 gm. of paraformaldehyde and the solution allowed to stand overnight. It was then poured onto ice and the carbinol extracted with ether, then distilled. B.p.  $115^\circ$  at 15 mm. Yield 31 gm. D  $\frac{27}{4} = 0.824$ .

$$[\alpha]_D^{27} = \frac{+0.67^\circ}{1 \times 0.824} = +0.81^\circ; [M]_D^{27} = +1.29^\circ \text{ (homogeneous)}$$

3.472 mg. substance: 9.674 mg. CO<sub>2</sub> and 4.320 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>O. Calculated. C 75.9, H 14.0

Found. " 76.0, " 13.9

*Dextro-2,5-Dimethyloctane*—31 gm. of 2,5-dimethyloctanol-8,  $[\alpha]_D^{27} = +0.81^\circ$ , were brominated by phosphorus tribromide as described for 7-bromo-2,5-dimethylheptane. This was not purified further than by distillation, due to the limited quantity.

A Grignard reagent was prepared from 3 gm. of magnesium in dry ether and 26 gm. of the above halide. This was poured onto ice and the hydrocarbon extracted with ether. It was purified as described for 2,5-dimethylheptane. It was then distilled from a small piece of sodium. B.p.  $156^\circ$ , at 760 mm. Yield 4 gm.

$$[\alpha]_D^{25} = \frac{+0.74^\circ}{1 \times 0.723} = +1.02^\circ; [M]_D^{25} = +1.45^\circ \text{ (homogeneous)}$$

3.932 mg. substance: 12.143 mg. CO<sub>2</sub> and 5.442 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>. Calculated. C 84.4, H 15.6

Found. " 84.2, " 15.5

## WINKLER TITRATION IN METHYLENE BLUE MEDIA

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In the course of some experiments by the writer it became convenient to determine dissolved oxygen by the Winkler method in a medium colored with methylene blue. It was found that the methylene blue served perfectly as an indicator on titration with thiosulfate, giving as definite and sharp an end-point as starch. Concentrations of the dye of about 0.00005 M were satisfactory. High concentrations made the end-point somewhat more difficult to judge. With practice, however, it was easily possible to obtain agreement among a series of samples to within 1 drop of 0.0125 M thiosulfate. Examination of the literature disclosed that Sinnat<sup>1</sup> had early employed methylene blue as an indicator with the same results in titrating iodine with thiosulfate. No record was found of a similar use of the dye in Winkler titrations. In view of the current interest in the effect of methylene blue on oxygen consumption under different conditions, the method may commend itself to other investigators.

Upon addition of  $MnCl_2$  and  $NaOH + KI$  the methylene blue is reduced to the colorless leuco compound; the color, however, gradually reappears, requiring about 2 minutes to regain its original intensity. When concentrated HCl is added the dye is converted to the sparingly soluble iodide;<sup>1</sup> a flaky precipitate is formed and the solution is colored a deep red-brown. Upon titration with sodium thiosulfate the iodide is changed back into methylene blue chloride, the mixture passing from a muddy red through muddy green to a clear blue as the precipitate disappears.

The question naturally arose as to just what part the methylene blue played in the reactions, for its reduction and subsequent

<sup>1</sup> Sinnat, F. S., *Analyst*, **35**, 309 (1910); **37**, 252 (1912).

reoxidation might conceivably introduce an error into the oxygen determinations. Three possibilities were considered: (1) The leuco dye might recover oxygen from the manganese; (2) the leuco dye might be oxidized by light; (3) catalytic decomposition of water might occur. In the latter two cases the oxygen equivalent of the methylene blue would have to be taken into account.

Light intensity within the range from strong sunlight to nearly complete darkness had no influence on the rate of recoloration. The concentration of dissolved oxygen was likewise without effect, between well saturated solutions and solutions saturated with rather impure hydrogen from a Kipp generator.

Titration of samples having the same concentration of dissolved oxygen but different concentrations of methylene blue showed the reoxidation to be effected by the manganese (possibility (1)). A typical run follows. Three bottles containing respectively Ringer's solution, Ringer's solution + 0.00005 M methylene blue, and Ringer's solution + 0.0001 M methylene blue were aerated vigorously for 30 minutes by a motor vacuum pump. A set of four Pyrex tubes (calibrated to 70 cc. volume) was filled from each bottle and carried through the Winkler routine. They were titrated with 0.025 M thiosulfate. Starch was employed as indicator for the Ringer solution, methylene blue for the other two. If the methylene blue contributed oxygen or an oxygen equivalent to the manganese, there should have been required per tube:

(a)	Ringer's solution.....	<i>x</i> cc. thiosulfate
(b)	" " + 0.00005 M methylene blue, <i>x</i> + 0.28 "	" "
(c)	" " + 0.0001 " " " + 0.56 " "	

Titration showed the following quantities (averages of four bottles).

(a)	Ringer's solution.....	3.03	cc. thiosulfate
(b)	" " + 0.00005 M methylene blue, 3.035 "	" "	"
(c)	" " + 0.0001 " " " 3.02 "	" "	"

Individual bottles varied by not more than 1 drop of 0.025 M thiosulfate (about 0.04 cc. with the particular burette used). The reduction of the dye, therefore, is completely reversed and introduces no error.

This behavior of methylene blue is interesting in itself. The oxidation of manganese by dissolved oxygen is usually considered

to be from the bivalent to the tetravalent state. Mixtures of the 2 ions give reversible oxidation-reduction potentials.<sup>2</sup> Although details at the high pH of the Winkler titration are lacking, it would seem that the Mn(OH)<sub>2</sub> formed in the initial reaction of MnCl<sub>2</sub> and NaOH would be strongly electronegative to the almost completely oxidized methylene blue. The latter, apparently, is reduced before appreciable amounts of tetravalent manganese are formed by reaction with dissolved oxygen. Formation of the tetravalent ion would cause a large increase in the potential. Evidently when this occurs, the relative positions of the manganese and methylene blue systems are reversed, the latter now being electronegative, and reoxidation occurs. Such an explanation would necessitate that the absolute velocity of the reaction between manganese and methylene blue be conspicuously greater than the velocity of oxidation of manganese by dissolved oxygen. And this disparity in velocities exists. The reduction of methylene blue occurs instantaneously upon dispersion of the manganeseous hydroxide throughout the tube, while the darkening of the hydroxide as it reacts with dissolved oxygen can be followed with the eye, even when pure oxygen gas is bubbled through the suspension.

<sup>2</sup> Grube, G., and Huberich, K., *Z. Elektrochem.*, **29**, 8 (1923).



## THE ABSORPTION OF CALCIUM SOAPS AND THE RELATION OF DIETARY FAT TO CALCIUM UTILIZATION IN THE WHITE RAT

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The influence of dietary fat on calcium soap formation and calcium absorption in the intestine has received considerable study with somewhat conflicting results. Cronheim and Müller (7), Bahrdt (3), Rothberg (18), and Birk (4) found that as the amount of fat in the diet increases above a moderate level calcium soaps in the stools also increase. When fatty acid utilization is low, as is the case with ethyl palmitate or palmitic acid, the loss of calcium soap is increased (Givens (9)). Bosworth, Bowditch, and Giblin (5) concluded that the presence of soluble ionized calcium in the intestine determines the extent of soap formation; while loss in the feces depends not only upon the amount of calcium soaps formed but also upon their nature, calcium oleate being much more soluble in the normal intestinal fluids than is calcium palmitate or calcium stearate. Telfer (22) also believes that the higher calcium content of cow's milk as compared with human milk is the factor that leads to the greater loss of calcium soaps in the stools of bottle-fed infants. Holt, Courtney, and Fales (13), on the other hand, concluded that a high calcium intake does not necessarily cause a large loss of fat in the feces. They found better assimilation of calcium by infants when the fat of the diet was generous than when it was more restricted.

Whether calcium soaps are lost in the feces in large quantities undoubtedly depends upon other factors in addition to the amount and nature of the calcium compounds and fats of the diet. One such factor is the acidity of the intestinal contents. Telfer (21) points out that free fatty acids dissolve calcium phosphate with

the precipitation of calcium soaps. If fats and phosphates both are low, calcium carbonate may be excreted in an alkaline stool. The form in which calcium is eliminated from the intestinal tract, therefore, is associated with the pH of the intestinal contents as a consequence of the effect of acidity upon solubilities.

The beneficial effects of dietary fat upon calcium assimilation as found by Holt and Fales (14) and the curative effect of vitamin-free fat upon rickets as found by Zucker and Barnett (24) probably depend, partly at least, upon a more favorable pH of the intestinal contents, produced by the fatty food. Boyd and Lyman have shown that the utilization of calcium soaps, particularly of calcium oleate, may be high, 70 to 80 per cent of the latter that is ingested being absorbed (6).

Haldane, Hill, and Luck (10) found that calcium chloride ingestion causes a loss of blood bicarbonate and a great increase in calcium carbonate of the feces. This observation has been verified by Gamble, Ross, and Tisdall (8) and by Atchley, Loeb, and Benedict (2). It thus appears that the chloride ion of calcium chloride is more rapidly absorbed than is its associated calcium ion. The calcium ions, therefore, associate with fatty acid, phosphate, or carbonate ions to form compounds that are but slightly soluble in the intestinal fluids. The particular compounds of calcium that form in any given experiment evidently will depend upon the products then present in the intestinal contents. Telfer (20) has stated these conditions thus: If fatty acids are in excess, the calcium is excreted as soap; if alkalinity predominates, calcium phosphate is formed; if both fats and phosphate are low in the diet, calcium may be excreted as carbonate. These facts can be put in another form: The lower fatty acids readily dissolve calcium carbonate and calcium phosphate. In the presence of higher fatty acids and their soluble soaps a secondary reaction may then result in the formation of insoluble calcium soaps and the liberation of carbonic and phosphoric acids (Telfer (19)). Stated in still another way, this is merely the effect of hydrogen ion concentration upon the solubility of salts of a strong base with weak acids.

Another well recognized factor in fat and calcium assimilation is the bile. It is well known that in the absence of bile from the intestine large quantities of fatty acids and calcium soaps are eliminated in the feces from fat-containing diets. That bile, and

solutions of bile salts in water, dissolve fatty acids and calcium soaps has been amply demonstrated (Verzár and Kúthy (23), Adler (1), Oelsner and Klinke (16)).

Our experiments are divided into two groups. The first group deals with the absorption of calcium soap; the second with the effect of dietary fat upon the assimilation of calcium.

#### *Utilization of Calcium Soaps*

*Method*—The general plan was to feed pure calcium soaps in a synthetic food mixture otherwise free, as far as practicable, from calcium and to determine the extent of utilization by a comparison of the soaps found in the feces of rats fed theron with the soap consumed with the food. The rats used were of Wistar stock, 35 days old, and weighing about 60 gm. each at the beginning of the test and capable of storing 30 to 40 mg. of calcium per rat per day. A preliminary period for adjustment to the diet was followed by a metabolism test of 17 to 20 days. For collection of urine and feces the rats were housed individually in galvanized wire cages over 12 inch glass funnels, provided with large Witt plates which retained the feces. The urine was collected in toluene-thymol preservative, containing dilute hydrochloric acid to prevent the precipitation of calcium salts. The feces were removed daily and the funnels rinsed down with water. Scattering of food was prevented by the use of a feeding cup devised from a 150 cc. beaker provided with a metal cover in whose center a hole 1 inch in diameter was punched. In order to feed, the rat was compelled to enter its head and shoulders into the cup.

The calcium-low basal ration consisted of special low calcium casein 18 gm., modified calcium-free Osborne and Mendel (17) salt mixture 4 gm., powdered sucrose 35 gm., starch 35 to 40 gm. To this mixture the calcium soap was added in the desired amount to make a total of 100 gm. 0.2 gm. of dried brewers' yeast and 2 drops of cod liver oil were given each rat daily as vitamin supplements.

The casein was decalcified by extraction with 0.01 N hydrochloric acid at 0°. Under these conditions there was no objectionable swelling. The calcium soaps were prepared according to Harrison's (11) method from palmitic, stearic, oleic, and butyric acids obtained from the Eastman Kodak Company. Mixed soaps from

the fatty acids of lard were prepared by the usual methods. Some difficulty was experienced from oxidative rancidity production in the preparation of calcium oleate. This was largely avoided by drying and keeping the product in a vacuum desiccator.

Calcium in urine, feces, and foods was determined by McCruden's (15) method in ashed material. The Roese-Gottlieb method as described by Holt, Courtney, and Fales (12) was used for determining soap, free fatty acids, neutral fat, and total fat in feces.

*Relative Absorption of Calcium*—When the level of calcium intake was relatively high, between 37 and 60 mg. a day per rat, absorption of calcium varied greatly among the different calcium compounds tested. Assuming that the calcium of the feces represents unabsorbed calcium, calcium utilization was as follows: lactate 68.5 per cent, butyrate 52.5 per cent, oleate 68.4 per cent, mixed soaps from lard 46.7 per cent, palmitate 39.4 per cent, and stearate 30.9 per cent. On comparing the soaps of the feces with the soap in the food, and assuming that, in feces, calcium soaps only are present, the following soap utilization values are found: oleate 90.4 per cent, mixed soaps 72 per cent, palmitate 38 per cent, and stearate 25 per cent. The higher utilization of calcium lactate as compared with butyrate probably depends upon the shift toward alkalinity produced by butyrate in the intestinal contents. It was noticed that the feces from butyrate-fed rats effervesced strongly when moistened with dilute hydrochloric acid. Most of the calcium in such feces was combined as carbonate, as shown by titration against acid with methyl orange indicator.

The relative absorption of calcium oleate, palmitate, and stearate is probably in the order of their solubilities in bile. The utilization of oleate is high, as was expected; but the utilization of stearate, while relatively low, is higher than was anticipated. When the soaps were fed at a lower level, enough to supply 13.6 to 21.4 mg. of calcium per rat per day, utilization of calcium was in the same order as that obtained at the higher level, but the percentage absorption was higher, 81 per cent of the oleate, 65 per cent of the palmitate, and 49 per cent of the stearate calcium having disappeared from the intestine. The utilization of the several soaps was oleate 91 per cent, palmitate 65 per cent, and stearate 45 per cent. In terms of mg., calcium absorption per rat per day varied from 17 mg. on the stearate ration to 40 mg. on the lactate

ration. Records of the calcium intake and losses of calcium are given in Table I.

*Absorption of Fatty Acid Radical*—If the calcium soaps are absorbed as undissociated molecules and no calcium is reexcreted into the bowel, there should be perfect agreement between the loss from the intestine of the fatty acid radical and that of the calcium atom. On the whole our results indicate that the soaps are absorbed without dissociation. Assuming that everything

TABLE I  
*Average Utilizations of Calcium in Calcium Soaps*

Kind of soap	No. of rats	Daily food intake	Daily gain in weight	Daily Ca intake	Daily Ca loss		Total daily Ca loss	Daily Ca storage	Ca absorbed	
		gm.	gm.	mg.	Feces	Urine			mg.	per cent
8 per cent palmitate...	8	8.3	2.3	53.6	32.5	1.4	33.9	19.7	36.6	21.1
8 " " "	8									39.4
+10 per cent lard...	3	8.7	3.1	47.1	20.6	1.2	21.8	25.3	53.4	26.5
4 per cent palmitate...	3	10.5	2.9	32.5	11.3	1.0	12.3	20.2	63.5	21.2
8 " " oleate.....	6	7.1	1.3	37.3	11.9	2.8	14.7	22.6	60.5	25.4
4 " " " .....	4	8.9	2.4	19.8	3.7	1.0	4.7	15.1	75.2	16.1
8 " " stearate....	3	9.5	2.6	56.3	39.1	1.7	40.8	15.5	27.5	17.2
4 " " " ....	4	9.9	2.5	28.3	14.4	0.9	15.3	13.0	46.3	13.9
8 " " butyrate...	4	8.7	2.3	103.5	58.3	12.4	70.7	32.8	31.8	45.2
4 " " " ...	3	10.0	2.4	60.0	28.5	2.1	30.6	29.4	49.0	31.5
3 " " lactate.....	7	9.8	2.4	58.2	18.3	2.4	20.7	37.5	64.3	39.9
8 " " mixed soaps.	6	8.5	2.3	54.9	29.3	2.6	31.9	23.0	43.5	25.6
8 " " " "	8									46.7
+10 per cent lard...	6	7.7	2.13	44.6	22.1	2.7	24.8	19.8	44.6	22.5
										50.3

All rats were given 0.2 gm. of yeast daily and 2 drops of cod liver oil. The average age of rats at beginning was 35 days; average weight 65 gm.

determined as soap in the feces is unabsorbed calcium soap from the feed and that no absorbed calcium is excreted via the bowel, the percentage utilization values shown in Table II are obtained.

In calculating the absorbability of the calcium of the soaps an uncertainty is introduced because of the presence in the diets of calcium other than that of the particular soap used. In some cases this other calcium amounted to as little as 3 per cent of the total, while in other cases it was as much as 22 per cent. This

## Utilization of Calcium Soaps

variability was due mainly to variations in calcium content of different lots of yeast and casein. In the experiments with the calcium soaps prepared from the fatty acids of lard, the calcium in the ration other than that combined as soap was relatively high, 17 to 22 per cent of the total; and in these experiments we had the poorest agreement between utilization of calcium and utilization of soap. Table III shows the fat-fatty acid analyses of the feces and the average utilization of the fatty acids of calcium soaps.

*Storage of Calcium*—Storage of calcium varied from 12.9 to 37.4 mg. of calcium per rat per day. In general, storage paralleled absorption since there was relatively little excretion of

TABLE II  
*Utilization of Calcium Soaps versus Utilization of Calcium*

Kind of soap	Ca soap intake mg.	Soap utilization per cent	Ca utilization
			per cent
8 per cent palmitate.....	47.0	38.3	39.4
8 " " " + 10 per cent lard.....	45.6	57.7	56.1
4 per cent palmitate.....	29.7	65.1	65.3
8 " " oleate.....	35.0	90.4	68.4
4 " " " .....	19.0	91.1	81.2
8 " " stearate.....	48.0	25.0	30.9
4 " " " .....	26.4	45.1	49.0
8 " " mixed soaps.....	43.1	72.1	46.7
8 " " " " + 10 per cent lard.....	36.6	67.2	50.3

calcium by way of the kidney except in one case in which calcium butyrate was fed at a high level.

*Effect of Dietary Fat upon Utilization of Calcium Soaps*—From a fat-free diet calcium palmitate was absorbed to the extent of 38.3 per cent. A similar diet containing lard to the extent of 10 per cent with a corresponding decrease in carbohydrate led to considerably greater calcium palmitate utilization; *viz.*, 57.7 per cent. In this experiment the effect of dietary fat upon calcium soap absorption was distinctly favorable. However, when we added lard to the fat-free diet whose calcium was supplied by the calcium soaps prepared from the mixed fatty acids of lard, there

was no marked effect on the utilization of the soap. We regard this experiment with the soaps from mixed fatty acids as less satisfactory than that with calcium palmitate because of the presence in the mixed calcium soap diet of considerable amounts of calcium in other forms than soap.

TABLE III  
*Average Utilization of Soap and Fatty Acids in Calcium Soaps*

Kind of soap	Calcium			Fatty acid						Neutral fat in feces daily	
				Daily soap intake			Daily soap intake				
	Daily soap intake	Daily soap loss in feces	Soap absorption	Daily soap intake	Daily soap loss in feces	Daily loss as free acid	Daily total loss	mg.	per cent		
mg.	mg.	per cent	mg.	mg.	mg.	mg.	mg.	mg.	per cent	mg.	
8 per cent palmitate . . . . .	47.0	28.8	38.3	598	368	25	393	205	34.2	13	
8 " " " +											
10 per cent lard . . . . .	45.6	19.2	57.7	578	244.9	26	270	308	53.3	16.8	
4 per cent palmitate . . . . .	29.7	10.4	65.1	378	132.0	24	156	222	58.7	19	
8 " " oleate . . . . .	35.0	3.4	90.4	490	47.4	10	57.4	432	88.1	12.6	
4 " " " . . . . .	19.0	1.7	91.1	265	24.0	4	28.5	247	89.5	6.1	
8 " " stearate . . . . .	48.0	35.8	25.0	673	502	41	543	130	19.3	23.0	
4 " " " . . . . .	26.4	14.5	45.1	370	203	28	231	139	37.4	15	
8 " " mixed soaps . . . . .	43.1	12.8	72.1	548	153	10	163	385	70.3	12	
8 " " " "											
+ 10 per cent lard . . . . .	36.6	12.1	67.2	466	154	11.6	166	300	64.2	10	

*Effect of Dietary Fat upon Absorption of Calcium Administered As Salts Soluble in Water*

The experimental method was, in general, the same as that already described in the first part of this paper. Food, fecal, and urinary calcium were determined as measurements of calcium absorption and storage. In this part of the work phosphorus determinations and pH measurements of the intestinal contents were made also.

The basal diet consisted of hydrochloric acid-extracted casein, containing about 0.014 per cent calcium as oxide, 18 per cent; modified Osborne-Mendel salt mixture, calcium-free, 3 per cent; starch 40 per cent; powdered sucrose 36 to 37.5 per cent; calcium lactate 3 per cent or calcium chloride, anhydrous, 1.5 per cent.

Each rat was given 0.2 gm. of dried brewers' yeast moistened with 2 drops of cod liver oil daily. The fat-containing diet was modified from the above by decreasing the starch to 35 per cent, the sucrose to 31 or 32.5, and adding 10 per cent of lard.

After preliminary adjustment to the fat-free diet, urine and feces were collected for 10 days. The diet was then changed to one containing fat, and collections continued 10 days longer, when the rats were killed and the acidity of the intestinal contents determined by means of the quinhydrone electrode. In making the pH determinations, dilution water was kept down to a volume about equal to that of the intestinal material used in the test. The Pemberton-Neuman method was used for phosphorus estimations in both urine and feces.

TABLE IV  
*Average Food Consumption, Gains in Weight, and Weight of Feces*

Ration	No. of rats	Aver-age initial weight gm.	Aver-age final weight gm.	Aver-age gain per day gm.	Aver-age weight of feces, 10 days gm.	Aver-age feed per day gm.
Ca lactate.....	4	68	97	3.1	2.03	11.8
" " + fat.....	4	97	115	1.8	2.13	8.6
" chloride.....	4	82	103	2.1	2.22	7.4
" " + fat.....	4	103	122	1.9	2.11	8.3

*Results*—Food consumption and gains in weight are given in Table IV. Gains were fairly good, ranging from about 2 to about 3 gm. per day. The animals appeared to be in good condition at all times.

*Effect of Dietary Fat upon Utilization of Calcium Chloride*—When calcium chloride supplied nearly all the calcium present in a fat-free diet, the calcium lost in the feces was 34 per cent of the intake, while 66 per cent was absorbed. After lard, to the extent of 10 per cent, was added to the ration, the loss of calcium in the feces fell to 22 per cent of the intake. After the addition of fat to the diet the loss of calcium in the urine was decreased nearly one-half. The amount of calcium stored per rat per day increased from 20.8 mg. of calcium on the fat-free diet to 29.3 mg. on the fat-containing diet. The addition of fat to a fat-free ration whose

calcium was supplied by calcium lactate showed a slight beneficial effect upon the absorption of calcium, but owing to a smaller food intake on the fat-containing diet, the storage of calcium was slightly lower after adding fat than it had been before. Table V gives the average daily data for calcium utilizations for each group of rats.

*Effect of Dietary Fat upon Soap Excretion When All the Calcium of the Diet Exists As Soluble Salt*—Table VI shows the amounts

TABLE V  
*Effect of Fat upon Utilization of Calcium*

Ration	No. of rats	Average Ca in feed, 10 days	Average Ca in feces, 10 days	Ca absorbed per cent	Ca in urine	Total Ca excreted	Total Ca stored per rat
		mg.	mg.	per cent	mg.	mg.	mg.
Ca lactate.....	4	410	104	75.0	12.5	116.5	293.5
" " + fat.....	4	366	84	77.0	5.7	89.7	276.3
" chloride.....	4	352	120	66.0	24.3	144.3	207.7
" " + fat.....	4	393	87	78.0	13.4	100.4	292.6

TABLE VI  
*Effect of Fat upon Carbonate and Soap Excretion*

Ration	No. of rats	Ca in feed, 10 days	Ca in feces, 10 days	Carbonates in feces as Ca	Total soaps in feces as fatty acids	Free fatty acids in feces	Neutral fat in feces
		mg.	mg.	mg.	mg.	mg.	mg.
Ca lactate.....	2	479	135	122	145	61	71
" " + fat.....	2	416	121	74	102	51	92
" chloride.....	2	461	191	162	116	51	131
" " + fat.....	2	513	159	99	52	41	86

of soaps, fatty acids, fat, and carbonate in the feces. The data in Table VI were obtained on only two of the four rats reported in Table V, hence the calcium intakes in the two tables are unlike. It is seen that on the fat-free diet the losses of carbonates and soaps in the feces are much greater than they are on a similar diet when fat is present. The effect of fat in reducing the fecal losses of carbonates and soaps is greater on calcium chloride than it is on calcium lactate. As we shall bring out later, this is in line

with the effect of fat upon increased intestinal acidity. That the rat is capable of digesting all the fat in a food mixture containing at least 10 per cent of fat is indicated by the fact that the losses of fecal free fatty acids and neutral fat are no greater on the fatty diet than upon the fat-free ration. Whatever soaps are produced in the digestive tract under the conditions of this experiment must be well absorbed, since actually less soap is excreted in the feces upon the fat-containing as compared with the fat-free diet. The amount of soaps resulting from both diets, however, was not large.

*Effect of Dietary Fat upon Utilization of Phosphorus*—Table VII shows that the addition of fat to a fat-free ration whose calcium is in the form of a soluble salt resulted in less loss of phosphorus in

TABLE VII  
*Effect of Fat upon Utilization of Phosphorus*

Ration	No. of rats	Aver-	Aver-	P ab-	P in	Total P ex-	P stored
		age P in feed, 10 days	age P in feces, 10 days	per cent	urine	creted	per rat
		mg.	mg.		mg.	mg.	mg.
Ca lactate . . . . .	2	587	67	89	81	148	439
" " + fat . . . . .	2	639	38	94	108	146	493
" chloride . . . . .	2	475	72	85	154	226	249
" " + fat . . . . .	2	530	22	96	158	180	350

the feces. This was more marked in the case of the calcium chloride ration, the fecal loss decreasing from 15 per cent of the intake on the fat-free ration to only 4 per cent when fat was present. This beneficial effect is undoubtedly due, partly at least, to the greater acidity of the intestinal contents on the fat-containing diet. The loss of phosphorus in the urine on the calcium chloride ration, both with and without fat, was high and not markedly changed by dietary fat. This large loss of phosphate in the urine is associated with the acidosis caused by calcium chloride.

*Effect of Fat upon Acidity of Contents of Stomach and Intestines*—Table VIII shows that the addition of fat to the calcium lactate, fat-free diet had but slight effect upon the pH of the contents of the stomach and intestines. In general there seemed to be a

slight shift toward alkalinity. The total effect of these shifts upon calcium and phosphorus absorption was not very pronounced. The effect of fat upon the reactions produced by the calcium chloride diet was a marked shift toward the acid except in the stomach where there was little change. Without fat in the diet calcium chloride produced a neutral or alkaline reaction in the lower half of the small intestine, in the cecum, and in the feces. In the upper half of the small intestine the shift was decidedly toward a more acid reaction as a result of the fat addition to the diet. These shifts in acidity as a result of adding fat to the diet seem to give

TABLE VIII  
*Effect of Fat upon Acidity of Digestive Tract*

Ration	No. of rats	Stomach, pH		Small intestine, pH			
				Upper half		Lower half	
		Range	Aver- age	Range	Aver- age	Range	Aver- age
Ca lactate.....	4	1.96-3.02	2.25	6.40-6.61	6.46	6.36-6.70	6.48
" " + fat.....	4	2.00-4.08	2.57	6.31-6.50	6.40	6.66-6.82	6.74
" chloride.....	4	2.26-4.65	3.89	6.14-6.98	6.44	6.73-7.25	6.98
" " + fat.....	4	3.42-4.87	4.26	5.46-6.07	5.81	5.97-6.74	6.49
<hr/>							
Cecum, pH							
<hr/>							
Ca lactate.....	4	6.00-6.74	6.44	6.58-6.98	6.76		
" " + fat.....	4	6.74-6.88	6.82	6.83-7.15	6.94		
" chloride.....	4	7.50-7.94	7.66	7.61-7.91	7.71		
" " + fat.....	4	6.51-6.99	6.70	6.58-7.16	6.82		

a good and sufficient explanation of the beneficial effects of fatty food upon the absorption of calcium and phosphorus when the diet contains all of its calcium as calcium chloride.

#### SUMMARY

Calcium soaps, in a fat-free diet, were fed to young white rats capable of storing from 30 to 40 mg. of calcium daily. On a calcium intake of 37 to 56 mg. per rat per day the following utilization values for the various soaps were found: calcium stearate 25 per cent, calcium palmitate 38 per cent, calcium oleate 90 per cent,

mixed calcium soaps from the fatty acids of lard 72 per cent. On a calcium intake of 20 to 32.5 mg. of calcium daily the utilization values were calcium stearate 45 per cent, calcium palmitate 65 per cent, and calcium oleate 91 per cent.

The addition of fat to a diet nearly all of whose calcium was supplied by calcium chloride (1) markedly increased the acidity of the contents of the small intestine, the contents of the cecum, and of the feces of rats fed thereon; (2) increased the absorption of calcium; (3) increased the absorption of phosphorus; (4) decreased the loss of carbonates in the feces; and (5) decreased the loss of soaps in the feces.

The effect of fat upon the acidity of the digestive tract and upon the absorption of calcium and phosphorus when the diet contained calcium lactate as the sole source of calcium was relatively slight.

The presence in the diet of moderate amounts of fat may have a beneficial influence on the absorption of calcium and phosphorus by maintaining a favorable acidity of the intestinal contents. The calcium soaps of palmitic and oleic acids that form in the intestine under such conditions are well utilized.

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## THE METABOLISM OF TRICAPRIN

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A previous paper by the author (1) reviewed the work which had been done on the nature of the depot fat resulting from diets containing particular fatty acid radicals, and showed that caprylic acid is deposited in traces but that the feeding of lauric acid results in a depot fat containing as much as 25 per cent lauric acid. Thus there was left only capric acid of the saturated fatty acid series to be studied, and the present paper is a report of the effect of feeding that acid to white rats.

### EXPERIMENTAL

The capric acid used was purchased from the Eastman Kodak Company. It had a melting point of 31.2°, a neutralization number of 320 (theoretical 326), and distilled at 153–155° (15 mm. pressure). The procedure followed was the same as in the previous experiments on tricaprylin and trilauryl. The triglyceride had a saponification number of 292 (theoretical 303.7). The diets are given in Table I.

In addition each rat was given separately each day a vitamin mixture composed of 0.1 gm. of dried spinach, 0.4 gm. of dried yeast, obtained from the Northwestern Yeast Company, and 0.01 gm. of oscodal (unsaponifiable fraction of cod liver oil).

The results of the experiment are given in Table II, including saponification and iodine numbers of the depot fat.

The results in Table II indicate that capric acid had been deposited in the depot fat. To determine the extent of its deposition, the solid acids were separated from the liquid acids by the lead salt-ether method. Neutralization and iodine numbers were determined as shown in the tabulation below.

# Metabolism of Tricaprin

## *Analysis of Fatty Acids of Rats on Diet D*

	Neutralization No.	Iodine No.	Per cent of total acids
Solid acids.....	220	7.4	48
Liquid " .....	221	64	52

TABLE I  
*Composition of Diets Administered*

	Diet A	Diet D
	per cent	per cent
Corn-starch.....	72	50
Cane-sugar.....	8	5
Salt mixture.....	5	5
Ether-extracted casein.....	15	15
Tricaprin.....		25

The salt mixture was that of Osborne and Mendel (2). The casein was extracted with ether for 3 days, the ether being changed each day.

TABLE II  
*Data for Rats on Various Diets*

	Rat No.	Initial weight gm.	Weight after fasting gm.	Loss in weight per cent	Final weight gm.	Days fed	Saponifi- cation No.	Iodine No.
Control rats on Diet A	201	206	160	24	207	28	195	60
	202	226	187	18	232	28	194	64
	203	172	131	23	192	20	194	62
	204	324	274	15	321	19	204	62
	205	256	200	22	248	19	200	64
	206	248	202	15	240	28	200	68
<b>Average.....</b>							$198 \pm 1$	$63.3 \pm 0.7$
Rats on Diet D (tricaprin)	207	310	245	21	292	27	215	52
	208	210	153	27	200	19	217	49
	209	210	162	23	208	19	217	45
	210	218	161	26	223	27	219	43
	211	240	181	25	234	28	212	56
	212	330	253	23	297	25	217	42
<b>Average.....</b>							$216 \pm 1$	$47.7 \pm 1.6$

It is apparent that most of the capric acid is combined with the oleic acid in the liquid acid fraction. Capric acid, calculated on the basis of the iodine number, comprises 29 per cent of the liquid acids, and calculated on the basis of the neutralization number, it comprises 25 per cent. Assuming that the solid acids contain, besides the small amount of oleic, only capric and palmitic acids, the per cent of capric acid is 3.7. On the basis of these determinations, the composition of the fat of the rats on tricaprin is given below. Unfortunately there was not enough material to isolate the capric acid in the liquid fraction by distillation.

	<i>per cent</i>
Capric acid.....	15
Palmitic acid.....	42
Oleic acid.....	43

#### DISCUSSION

When butyric or caproic acid is fed to rats, it has been impossible thus far to demonstrate the presence of the acids in the depot fat (3, 4). Caprylic acid is found in traces (1). Lauric acid is found in amounts as high as 25 per cent (1) and myristic acid to the extent of 17.5 per cent (3). In the present instance, capric acid comprised 15 per cent of the depot fat, indicating that acids with chains of 8 carbon atoms or less tend to disappear or to be changed into longer chains, while acids with chains of 10 or more carbon atoms are deposited in considerable amounts without change.

#### SUMMARY

When tricaprin is the only fat fed to white rats, capric acid comprises 15 per cent of the fatty acids of the depot fat.

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## STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS

### X. THE SOLUBILITY OF HEMOGLOBIN IN SOLUTIONS OF CHLORIDES AND SULFATES OF VARYING CONCENTRATION\*

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#### INTRODUCTION

The solubility of proteins in aqueous solutions of neutral salts has formed one basis for their classification and characterizations. Thus solubility of one class of proteins, the globulins, is increased by the addition of small amounts of salt and decreased in more concentrated solutions of electrolytes. Albumins, on the other hand, are relatively soluble in water, but their solubility, also, is decreased in sufficiently concentrated solutions of certain salts. The increase in solubility of certain proteins on the addition of neutral salts was discovered in 1856 by Denis (10). This property was subsequently used in effecting separation between proteins, but it was not until 1905 that an exhaustive study of the solubility of globulins in dilute salt solutions was reported. In that year appeared the classical papers of Hardy (14) and of Mellanby (17) upon serum globulin and of Osborne and Harris (18) upon edestin. There have since been many other contributions to this descriptive chemistry of the proteins.

The theoretical explanation of the effects of electrolytes upon proteins awaited, of necessity, the development of an adequate theory of strong electrolytes. The classical theory of Arrhenius,

\* A preliminary report of a part of this investigation was presented at The Thirteenth International Physiological Congress at Boston, August, 1929 (13).

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which depended upon the "degree of dissociation," and, therefore, upon the number of particles present, neglected the electrical interaction of ions. Differences in the effect of different salts, especially of those of different valence types, on protein behavior were studied by Mellanby (17) who described in the following terms the solubility of globulins in salt solutions.

"Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of their valencies."

This statement of Mellanby is now recognized as a description of the principle of the ionic strength which was later formulated by Lewis (16). In studying mixtures of electrolyte solutions the latter found the following empirical relation. "In dilute solutions, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength."

The ionic strength does not explain differences in behavior between salts of the same valence type manifested in more concentrated solutions. The deviations from the ideal gas laws exhibited by solutions of strong electrolytes have been described in terms of the "activity coefficients" of the salts. The theory of Debye and Hückel (9) considered the influence of interionic forces upon activity coefficients. These equations have been found to be in close agreement with the experimental observations for many classes of strong electrolytes. The applicability of this equation to proteins has been considered by Cohn and Prentiss (8), by Stadie (22), and by Adair (1).

The two preceding papers in this series have dealt with the solubility of hemoglobin in concentrated solutions of different electrolytes under varying conditions (11) and in potassium phosphate buffers of varying concentration and pH (12). In this paper we consider the solubility of the same protein in solutions of chlorides and sulfates of widely varying concentration, especially in dilute solutions where their solvent effect can be investigated.

#### EXPERIMENTAL

The method of determining solubility is essentially the same as that employed in the foregoing papers. The crystallized horse

hemoglobin used was prepared as far as possible in the salt solution in which solubility was later determined. The crystals were placed in covered containers, the desired electrolyte solution added, the mixture saturated with carbon monoxide, and then shaken gently at 25° in a constant temperature bath until equilibrium was reached; generally 4 or 5 hours were required. The solutions were filtered at constant temperature and the filtrates analyzed. The crystals were returned to their containers and more electrolyte solution added. Most of the points on the sodium sulfate curve were obtained by repeated washing with a solution of a given concentration until constant solubility was reached. The same procedure was used in studying solubility in chlorides, but the solubility is so high in concentrated chloride solutions that it was not possible to obtain filtrates of constant salt content, but true equilibrium was reached since the points all lie on a smooth curve. In determining solubility in the rest of the electrolytes two bottles of crystals were used and the salt concentration was first increased from equilibrium to equilibrium and finally decreased in the successive equilibrations. The fact that the earlier and later points all lie on the same smooth curve and also that the points in the more concentrated solutions check those obtained in the previous series of experiments indicate that true equilibrium was reached.

The amount of hemoglobin dissolved was determined, as before, by Kjeldahl nitrogen analyses on the filtrates. Horse hemoglobin is assumed to contain 17.7 per cent of nitrogen. The sulfate solutions were analyzed for salt by heat-coagulating the protein without the addition of further salt, washing the precipitate salt-free, and carefully evaporating, drying, gently igniting, and weighing the filtrate. The magnesium sulfate determinations were checked by transforming a few samples to barium sulfate and weighing as such. The ammonium sulfate concentration was, however, determined by heat-coagulating the protein in the presence of a slight amount of phosphate buffer, washing the protein salt-free, and distilling over the ammonia in the filtrate. The chloride determinations were made according to the Wilson modification of the Van Slyke method (23).

The density of many of the saturated solutions was determined and graphical interpolation employed where necessary.

TABLE I  
*Solubility of Carboxyhemoglobin at 25°*

Experiment No.	No. of determinations	Concentration of electrolyte	Density	$\mu$	$\sqrt{\mu}$	pH	Hb solubility	Log solubility per 1000 gm. H <sub>2</sub> O
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Solubility in potassium chloride

		<i>mols per l.</i>					<i>gm. per l.</i>	<i>gm. per 1000 gm. H<sub>2</sub>O</i>	
20	1	0.108		0.114	0.338	6.62	61.2	64.4	1.809
20	1	0.110	1.020	0.116	0.341	6.59	62.8	66.3	1.821
20	1	0.258		0.281	0.537	6.59	100.0	109.0	2.037
20	1	0.272		0.297	0.545	6.69	101.0	112.0	2.049
20	1	0.374		0.415	0.644	6.64	123.0	136.5	2.135
20	1	0.425	1.051	0.474	0.689	6.69	124.0	138.6	2.142
20	1	0.614		0.701	0.837	6.65	145.0	165.6	2.219
20	1	0.698		0.807	0.899	6.65	150.0	173.2	2.238
20	1	0.807		0.943	0.971	6.65	163.0	190.0	2.279
20	1	0.970	1.083	1.15	1.07	6.64	160.0	189.6	2.278
20	1	1.002	1.067	1.19	1.09	6.62	159.0	188.8	2.276
20	1	1.040		1.23	1.11	6.66	160.0	190.5	2.280
20	1	1.295		1.565	1.25	6.66	170.0	206.0	2.314
20	1	1.63		2.0	1.41	6.74	163.0	199.5	2.300
20	1	1.76	1.112	2.16	1.47	6.60	166.0	204.0	2.310
20	1	1.94		2.395	1.545	6.61	159.0	196.5	2.293
20	1	2.70	1.150	3.34	1.83	6.76	140.0	173.2	2.238
20	1	3.20	1.169	3.90	1.98	6.62	110.0	134.0	2.127

Solubility in sodium chloride

		<i>mols per l.</i>		<i>gm. per l.</i>	<i>gm. per 1000 gm. H<sub>2</sub>O</i>				
16	1	0.004		0.004	0.064	6.60	24.2	24.7	1.393
16	1	0.114	1.021	0.121	0.348	6.54	62.8	65.9	1.820
16	1	0.117		0.123	0.351	6.66	67.8	71.2	1.852
16	1	0.242		0.264	0.514	6.76	100.0	109.0	2.039
16	1	0.249	1.0340	0.272	0.522	6.53	105.0	115.0	2.061
16	1	0.525	1.0612	0.600	0.776	6.66	155.0	177.0	2.248
16	1	0.53		0.604	0.778	6.70	157.0	179.0	2.253
16	1	0.55		0.622	0.79	6.81	163.0	186.0	2.269
16	1	1.05		1.25	1.12	6.69	198.0	235.0	2.37
16	1	1.60	1.1386	1.95	1.40	6.75	226.0	276.0	2.441
16	1	2.20		2.79	1.67	6.71	240.0	304.0	2.483
16	1	2.46	1.1650	3.195	1.79	6.61	248.0	319.0	2.504
16	1	2.83		3.72	1.93	6.80	254.0	334.0	2.526
16	1	3.10	1.1895	4.23	2.06	6.83	277.0	379.0	2.579

TABLE I—*Concluded*

Experiment No.	No. of determinations	Concentration of electrolyte <i>moles per l.</i>	Density	$\mu$	$\sqrt{\mu}$	pH	Hb solubility	Log solubility per 1000 gm. $H_2O$	
Solubility in potassium sulfate									
21	1	0.0268	1.0119	0.0831	0.283	6.73	41.4	42.8	1.631
21	1	0.0673		0.210	0.458	6.72	49.0	51.0	1.708
21	1	0.0904		0.283	0.532	6.77	53.0	55.2	1.742
21	1	0.1064	1.0263	0.333	0.577	6.80	48.6	50.7	1.705
21	1	0.1155		0.361	0.601	6.38	46.1	47.7	1.679
21	1	0.1760		0.551	0.743	6.60	49.0	51.2	1.709
21	1	0.205		0.642	0.801	6.70	43.3	45.2	1.655
21	1	0.322	1.0501	1.005	1.003	6.67	37.3	39.0	1.591
21	1	0.403		1.26	1.12	6.90	25.7	26.9	1.430
21	1	0.490	1.0669	1.53	1.24	6.45	20.5	21.3	1.328
21	1	0.520	1.0644	1.62	1.28	6.60	13.3	13.85	1.142
Solubility in sodium sulfate									
22	1	0.006		0.018	0.13	6.72	30.2	30.9	1.490
24	1	0.015		0.045	0.21	6.71	31.1	31.9	1.504
22	2	0.0523	1.0153	0.156	0.404	6.70	45.0	46.9	1.671
23	2	0.141		0.446	0.667	6.84	48.1	50.6	1.704
24	1	0.133		0.420	0.648	6.68	43.8	46.1	1.664
15	3	0.30		0.940	0.970	6.71	43.7	46.5	1.668
15	3	0.40	1.0576	1.265	1.125	6.66	32.1	33.8	1.529
15	2	0.55	1.0735	1.69	1.30	6.68	17.3	17.7	1.248
11	5	0.675	1.0816	2.12	1.455	6.60	9.56	9.78	0.990
Solubility in ammonium sulfate									
21	1	0.058	1.0120	0.180	0.424	6.41	41.8	43.2	1.636
21	1	0.205	1.0300	0.648	0.805	6.64	54.9	57.8	1.762
21	1	0.239		0.748	0.865	6.45	49.4	52.1	1.717
21	1	0.267		0.846	0.92	6.54	54.4	57.5	1.760
21	1	0.387		1.23	1.11	6.58	45.2	47.8	1.679
21	1	0.572		1.82	1.35	6.52	38.4	40.8	1.611
21	1	0.621		1.98	1.41	6.59	26.0	27.6	1.441
21	1	0.645	1.0507	2.05	1.43	6.60	25.1	26.7	1.427
Solubility in magnesium sulfate									
21	1	0.0652	1.0186	0.272	0.522	6.66	52.0	54.2	1.734
21	1	0.177	1.0411	0.756	0.87	6.59	83.0	88.6	1.947
21	1	0.339	1.0643	1.46	1.21	6.54	96.0	103.5	2.015
21	1	0.478		2.05	1.43	6.64	87.0	93.6	1.971
21	1	0.862	1.1120	3.63	1.91	6.60	62.2	65.3	1.815
21	1	0.881	1.1159	3.69	1.92	6.41	55.6	58.3	1.766
21	1	1.02	1.1251	4.23	2.06	6.49	47.9	49.7	1.696
21	1	1.77		7.25	2.69	6.1	8.25	8.43	0.926

The pH of all saturated solutions was determined with the hydrogen electrode as soon as possible after filtering. The hemoglobin and the electrolyte solutions were adjusted as nearly as possible to pH 6.6 before the experiment began, and where necessary small quantities of acid or alkali were added to the electrolyte solutions during the experiment. The pH values as determined lie for the most part between 6.5 and 6.8 in the range of minimum solubility. The data are presented in Table I. The ionic strength term of Lewis,  $\mu$ , is calculated per 1000 gm. of H<sub>2</sub>O.

*Activity Coefficients of Hemoglobin in Solutions of Chlorides and Sulfates*

In a heterogeneous equilibrium in which a solution is saturated with a solid component, at constant temperature and pressure, the activity of the substance in the solid phase must be the same as that in the liquid phase. This is independent of any other components in the solution. The addition of electrolytes to a saturated solution of hemoglobin does not change the activity of the hemoglobin although it changes the solubility, or the concentration. The ratio of the solubility of a substance at zero electrolyte concentration,  $S_0$ , to the solubility in a solution of given electrolyte concentration,  $S$ , is the activity coefficient,  $\gamma$ , of the substance in that solution.  $\gamma = \frac{S_0}{S}$ .

The Debye-Hückel theory of interionic forces in solutions of strong electrolytes yields a simplified equation for the evaluation of the activity coefficients in various solutions of electrolytes. The application of this theory to oxyhemoglobin in phosphate solutions at 0° has already been discussed by Cohn and Prentiss (8). The simplified form of the Debye equation including the "salting out" term added by Hückel is

$$-\log \gamma = \log S - \log S_0 = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A \sqrt{\mu}} - K_s \mu \quad (1)$$

in which  $\gamma$  is the activity coefficient,  $S$  is the solubility,  $S_0$  the solubility in the absence of electrolytes,  $\mu$  the ionic strength,  $Z_1 Z_2$  the valence type,  $K_s$  the "salting out" constant, 0.5 a theoretical constant having this value at 25°,  $A$  a constant which depends upon the mean effective diameter,  $b$ , of all ions in a solution, and  $\kappa$  a "reciprocal distance."

When the number of ions in solution is so small that their size is negligible in comparison with the distance between them they may be considered as point charges and Equation 1 reduces to

$$-\log \gamma = \log S - \log S_0 = 0.5 Z_1 Z_2 \sqrt{\mu} \quad (2)$$

This equation has been shown to hold for cobaltamines (3) and other difficultly soluble strong electrolytes in very dilute salt solutions. The molecular concentration of the cobaltamines is in general such that their contribution to the ionic strength is of the order of  $5 \times 10^{-4}$ . Thus their solubility could be studied in solutions of low ionic strength and it was possible to accurately determine  $S_a$ , the solubility in the absence of any electrolyte, rather than  $S_0$ , the solubility in the absence of added electrolyte. The solubility of hemoglobin in the absence of salt is relatively high, about 20 gm. per liter. The molecular concentration, however, is only 0.0003 M and of the same order as that of the cobaltamines. The contribution of the hemoglobin to the electrical environment remains unknown and in all of our calculations has been ignored. Moreover, difficulties of various kinds were encountered in making the measurements in very dilute solutions of salt. The results were less consistent and greater difficulty was experienced in controlling and measuring the hydrogen ion activity.

When the number of ions is increased their mean effective diameter must be taken into account. The right-hand side of the equation is then multiplied by the factor  $\frac{1}{1 + A \sqrt{\mu}}$ . This term changes the equation to

$$-\log \gamma = \log S - \log S_0 = \frac{0.5 Z_1 Z_2}{1 + A \sqrt{\mu}} \quad (3)$$

the equation of a curve rather than that of a straight line. This is the equation used by Cohn and Prentiss (8) to describe the solubility of horse oxyhemoglobin at 0°, in phosphate buffers up to an ionic strength of 1.0.

A third term must be added when more concentrated solutions of electrolytes are considered. Deviations of the behavior of strong electrolytes from the equation that Debye advanced in 1923 were subsequently ascribed by Hückel (15) to changes in the dielectric constant of the solution and shown to be approximately

proportional to the concentration. The added proportionality factor,  $K_s$ , is the so called "salting out" constant in Equation 1.

The solubility of hemoglobin in concentrated solutions of multi-valent electrolytes may be adequately described, as was seen in Studies VIII (11), by the linear equation for the solubility of non-electrolytes (20) and proteins in concentrated salt solutions (5)

$$\log S = \beta - K'_s \mu \quad (4)$$

where  $S$  is again the solubility and  $\mu$  the ionic strength.  $\beta$  is the intercept constant and is the hypothetical solubility in the absence of salt, termed hypothetical since, of course, hemoglobin is a globulin whose solubility passes through a maximum with increasing salt concentration.  $\beta$  is nevertheless a convenient constant varying with the temperature and the amphoteric properties of the protein.  $K'_s$  is a salting out constant characteristic of the protein independent of the temperature and of the pH when a given electrolyte is employed, but varying with the electrolyte. The evaluation of the constants  $K'_s$  and  $\beta$  affords an adequate characterization of the precipitation of a protein in concentrated solutions of electrolytes.

In studying the solubility of carboxyhemoglobin in phosphate buffers of varying ionic strength and pH (12) it was found that at the point of minimum solubility the protein acted as though it were composed not only of the neutral molecule, but of positively and negatively charged molecules as well. It was also found that the pH of minimum solubility varied with the salt concentration. The magnitude of the effect of electrolytes on the acid and basic dissociation constants was such that the proportion of charged to uncharged molecules at pH 6.6 was approximately constant, although slightly greater in the very dilute solutions of phosphate. Thus, although the slope of the solubility curve at pH 6.6 in dilute solutions was such that the Debye-Hückel equation did not appear to apply, if one considered the neutral molecule alone, it was found to act as though  $A$  in Equation 1 were 1.5, the value used in describing the phosphate buffers themselves (4), and  $Z_1Z_2$  were 4, the value used describing solubility in phosphates at 0°. The variation in the dissociation constants was such that if the solubility had been determined at pH 6.7 rather than 6.6 the protein would have consisted of an approximately constant proportion of charged to uncharged particles.

The pH of the saturated solutions of hemoglobin in sulfates and chlorides was not so accurately controlled as in the phosphate buffers. The points lie for the most part on smooth curves, however, and the variation in pH is not sufficient, apparently, to take the protein far from the pH of minimum solubility.

A satisfactory picture of the variation of activity coefficients can be obtained from a study of variation of solubility at approximately pH 6.6. The consideration of Equations 1 and 4 in relation to the solubility of hemoglobin in phosphate buffer solutions revealed the applicability of the first to dilute solutions and of the second to concentrated solutions. Equation 4 has been shown to be applicable to hemoglobin in concentrated sulfate solutions (11). The applicability of Equation 1 to solubility in both chlorides and sulfates is considered in the following.

#### *Apparent Valence Type, $Z_1Z_2$*

The "valence" type is evidently "apparent" when one is dealing with a complicated protein molecule. Hemoglobin has been found to behave as though it were a bi-bi- or a uni-quadrivalent type in the Debye sense. Consideration of the solubility of oxyhemoglobin in relation to the Debye equation led Cohn and Prentiss (8) to the following conclusions.

"The solvent action of a neutral salt upon a protein, oxyhemoglobin, has been found identical to the solvent action of a neutral salt upon a bi-bivalent or a uni-quadrivalent compound."

"To conclude that oxyhemoglobin is bivalent or quadrivalent might be correct, but would be unjustified at the present time. . . . Our experiments permit neither deduction. They render it certain, however, that oxyhemoglobin behaves in this respect as though it were bivalent or quadrivalent, and that the action of neutral salts in dissolving proteins is identical to their action in dissolving other slightly soluble substances."

The neutral carboxyhemoglobin molecule in solutions of phosphate behaves as though it had the same apparent valence type (12).

In chloride solutions it is possible to estimate the value of the apparent valence type since the salting out effect of chloride is relatively small, and Equation 2 may, in dilute solutions, be solved by simultaneous equations for  $Z_1Z_2$  and  $S_0$  if the value of  $A$  be taken from the literature. We have employed the value given by Scatchard (19). The results are reported in Table II.  $Z_1Z_2$  is 4.

It must be reiterated that *on the basis of this equation* hemoglobin merely acts *as though* it had this apparent valence type.

### Evaluation of $S_0$ . Solubility of Carboxyhemoglobin in Absence of Salt

The estimation of  $S_0$  is an extrapolation and its accuracy is dependent upon the applicability of the equation by which the extrapolation is made. On the basis of the calculation by Equa-

TABLE II  
Apparent Valence Type and Solubility in Absence of Salt of Carboxyhemoglobin at 25° and pH 6.6 Estimated by the Equation

$$\log S = \log S_0 + \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A \sqrt{\mu}}$$

$\log S$		$0.5 Z_1 Z_2$	$\log S_0$
Sodium chloride; $A$ taken as 0.775			
1.393	0.004	2.10	1.282
1.820	0.121	2.11	1.288
1.852	0.123	2.07	1.300
2.039	0.264	1.97	1.304
2.061	0.272	1.95	1.318
2.248	0.600	1.85	1.280
2.253	0.604	1.89	1.283
2.269	0.622	1.92	1.290
Potassium chloride; $A$ taken as 0.816			
1.809	0.114	2.05	1.280
1.822	0.116	2.02	1.288
2.037	0.281	1.99	1.289
2.049	0.297	1.99	1.294
2.135	0.415	1.94	1.290
Average.....		1.99	1.291

tion 2, from the solubility in dilute chloride solution reported in Table II, the minimum solubility of hemoglobin in the absence of salt is 19.5 gm. per liter.

The solubility of the neutral form alone in the absence of salt as determined by extrapolation from the data for solubility in phosphate solution is 11 gm. per liter. If the increase in solubility is

defined by Equation 9 of Studies IX and the values of  $pK_1K_2$  and  $pK_3K_4$  given there be accepted, the total solubility at pH 6.6 is about 17 gm. per liter, but the minimum solubility at pH 6.8 is only about 15 gm. per liter.  $\log S_0$  at the pH of minimum solubility, then, is 1.29 by extrapolation from the data for solubility in chloride, and 1.18 from the data for solubility in phosphates. The discrepancy may be due either to experimental error, especially in the unbuffered chloride solutions, or to estimation of the dissociation constants in dilute solutions of phosphates. In any case the true value of  $\log S_0$  is probably  $1.24 \pm 0.06$ . In considering the solubility in sulfates the value 1.20 makes it possible to more accurately describe the data in terms of the other constants of Equation 1. In Fig. 1 the curves are drawn with this extrapolated value.

#### *Calculation of the Salting Out Constant, K.*

Hemoglobin is not precipitated in the most concentrated solutions of sodium or potassium chloride. In sodium chloride there is not even a diminution of solubility. However, the solubility curve in potassium chloride does exhibit a maximum. No such curve can be described on the basis of the original Debye equation. It is necessary to employ a salting out constant as was done by Hückel. The calculation of this constant is carried out in Table

III. The term  $\log S - \frac{2\sqrt{\mu}}{1 + A \sqrt{\mu}}$  diminishes in more concentrated solutions. If this deviation from the Debye equation be divided by the ionic strength a constant value results. The deviation is given in the fourth column of Table III and is 1.30

$-\log S + \frac{2\sqrt{\mu}}{1 + A \sqrt{\mu}}$ .  $K_s$  for carboxyhemoglobin at  $25^\circ$  and pH 6.6 in sodium chloride solutions is 0.09 and in potassium chloride solution is 0.15.

In concentrated solutions of electrolyte in which the protein is precipitated, the solubility is described by Equation 4. If, as previously pointed out (7), Equations 1 and 4 apply simultaneously,  $\beta$  replaces  $\log S_0 + \frac{0.5 Z_1Z_2 \sqrt{\mu}}{1 + A \sqrt{\mu}}$  and  $K_s'$  is  $K_s$ . As a first approximation let us assume the applicability of the  $K_s'$  value for sulfates given in Studies VIII.

TABLE III

*Salting Out Constant for Carboxyhemoglobin in Solutions of Sodium and Potassium Chloride Estimated by the Equation*

$$1.30 - \log S + \frac{2\sqrt{\mu}}{1 + A\sqrt{\mu}}$$

Log $S$	$\mu$	$\frac{\log S -}{2\sqrt{\mu}} \frac{2\sqrt{\mu}}{1 + A\sqrt{\mu}}$	$\frac{1.30 - \log S +}{2\sqrt{\mu}} \frac{2\sqrt{\mu}}{1 + A\sqrt{\mu}}$	$K_s$
Sodium chloride; $A$ taken as 0.775				
2.370	1.25	1.170	0.130	0.104
2.441	1.95	1.100	0.200	0.103
2.483	2.79	1.029	0.271	0.097
2.504	3.20	1.007	0.293	0.092
2.526	3.72	0.981	0.319	0.086
2.579	4.29	0.991	0.309	0.075
Average.....				0.093
Potassium chloride; $A$ taken as 0.816				
2.278	1.15	1.136	0.164	0.143
2.276	1.19	1.124	0.176	0.148
2.280	1.23	1.117	0.183	0.149
2.314	1.57	1.077	0.223	0.144
2.300	2.00	0.988	0.312	0.156
2.310	2.16	0.974	0.326	0.151
2.293	2.40	0.926	0.374	0.156
2.239	3.34	0.769	0.531	0.159
2.127	3.90	0.612	0.688	0.176
Average.....				0.154

TABLE IV

*Values of  $A$  and  $K_s$  for Carboxyhemoglobin at 25° When Log  $S_0$  Is 1.20 and  $Z_1Z_2$  Is 4*

Electrolyte.....	NaCl	KCl	MgSO <sub>4</sub>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	K <sub>2</sub> SO <sub>4</sub>	Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub>
$K_s$ .....	0.12	0.27	0.31	0.56	0.72	0.72	0.65
$A$ .....	0.6	0.5	0.7	0.8	0.9	0.9	0.9

*Estimation of A*

The values of  $A$  used are purely empirical. If the values of  $K'$  calculated in Studies VIII are employed and if  $\log S_0$  be taken as 1.20 and  $Z_1Z_2$  be assumed to be 4, the values of  $A$  which best describe the experimental points can be calculated and are given in Table IV. In order to obtain uniformity, the curves describing solubility in chlorides are calculated assuming  $\log S_0$  to be 1.20 rather than 1.29 as calculated in Table II. This necessitates the

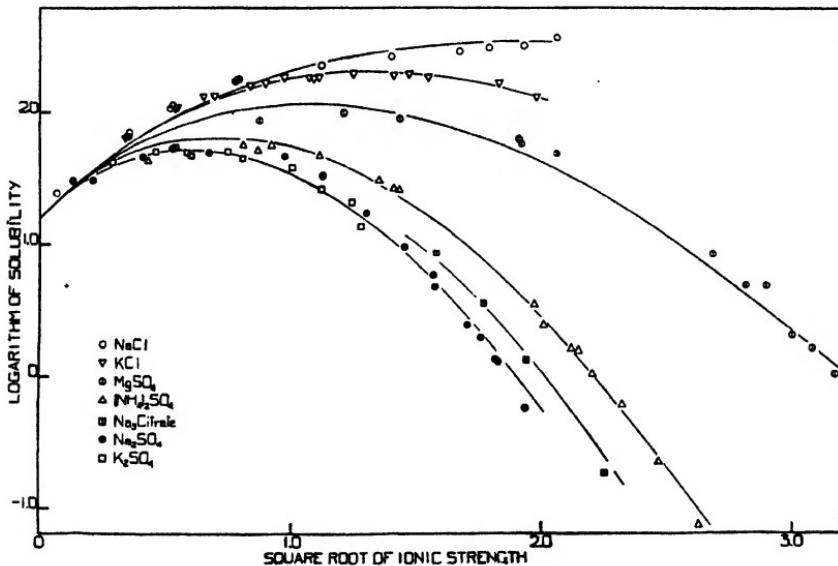


FIG. 1. The solubility of carboxyhemoglobin in various electrolytes at 25°. The curves are drawn according to Equation 1, the constants in Table IV being used.

use of a smaller purely empirical value of  $A$ . The agreement of the data with the equation can be seen in Fig. 1 where we have plotted  $\log S$  against  $\sqrt{\mu}$ .

There are ranges in which there is a divergence from the calculated curves, notably in the dilute concentrations of ammonium and magnesium sulfates and in sodium sulfate at about  $\sqrt{\mu} = 1.3$ . The studies upon phosphates considered in the previous communication are also not satisfactorily described by Equation 1. Although this equation describes a decrease in activity coefficient

followed by an increase in activity coefficient, the rates of decrease and increase, especially in the neighborhood of maximum solubility, are different for carboxyhemoglobin than for  $MgSO_4$  or for a cobaltamine.

#### *Empirical Equations for Description of Solubility*

The solubility of hemoglobin in solutions of chlorides and sulfates may be satisfactorily described in terms of an empirical equation which is similar in form to the Debye-Hückel equation if the size term be omitted, which may then be written:

$$-\log \gamma = \log S - \log S_0 = 0.5 Z_1 Z_2 \sqrt{\mu} - K_s \mu \quad (5)$$

This equation is the same as that used by Bjerrum and Unmack (2) in characterizing the activity coefficients of various electrolytes. We have changed the symbols for convenience, and written:

$$-\log \gamma = \log S - \log S_0 = k_i \sqrt{\mu} - k_o \mu \quad (6)$$

in which  $k_i$  and  $k_o$  are empirical constants,  $k_i$  being the "salting in" constant and  $k_o$  the "salting out" constant.  $k_i$  may be assumed to be the "apparent valence type" replacing  $0.5 Z_1 Z_2$  in the Debye equation. The term  $\frac{1}{1 + A \sqrt{\mu}}$  is omitted and  $k_o$  is substituted for  $K_s$ , for although both are salting out constants they have not the same numerical value. The values of both constants vary with the electrolyte. If one were to attempt a comparison of this equation with that of Debye and Hückel, this variation of  $k_i$ , the "apparent valence type," ( $0.5 Z_1 Z_2$ ) might seem improbable but Simms (21) has reported a similar phenomenon in describing the activity coefficients of amino acids and other weak electrolytes and amphotytes. However, we used this equation merely as an empirical interpolation formula and its justification is the greater accuracy with which the activity coefficients are described.

One may use a still simpler expression involving the equivalent concentration,  $C$ , of electrolyte rather than the ionic strength,  $\mu$ . In this case the equation becomes

$$\log S = \log S_0 + k_i \sqrt{C} - k_o C \quad (7)$$

Here, fortunately,  $k_e$  has the same value, 1.6, for all electrolytes and  $\log S_0$  is 1.30. The equation may be rearranged and these values substituted whence

$$\log S - 1.6 \sqrt{C} = 1.30 - k_e C \quad (8)$$

If  $\log S - 1.6\sqrt{C}$  be plotted against the equivalent concentration of electrolyte,  $C$ , a straight line results, describing the solubility in

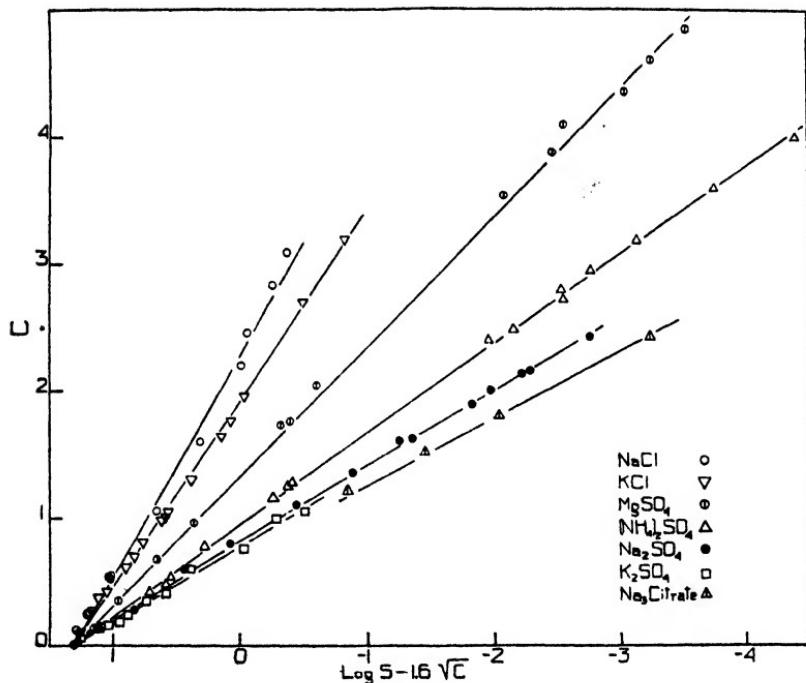


FIG. 2. The solubility of carboxyhemoglobin at 25°. The lines are drawn according to Equation 8.

any given electrolyte. The slope of the line is  $k_e$  and varies with the electrolyte used having the following values for carboxyhemoglobin of the horse at 25°: NaCl, 0.57; KCl, 0.67; MgSO<sub>4</sub>, 0.98; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.40; Na<sub>2</sub>SO<sub>4</sub>, 1.64; K<sub>2</sub>SO<sub>4</sub>, 1.68; and Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, 1.87. These values have been determined graphically from Fig. 2 where the agreement with the experimental points may be seen to be satisfactory. The solubility in potassium phosphates at pH 6.6

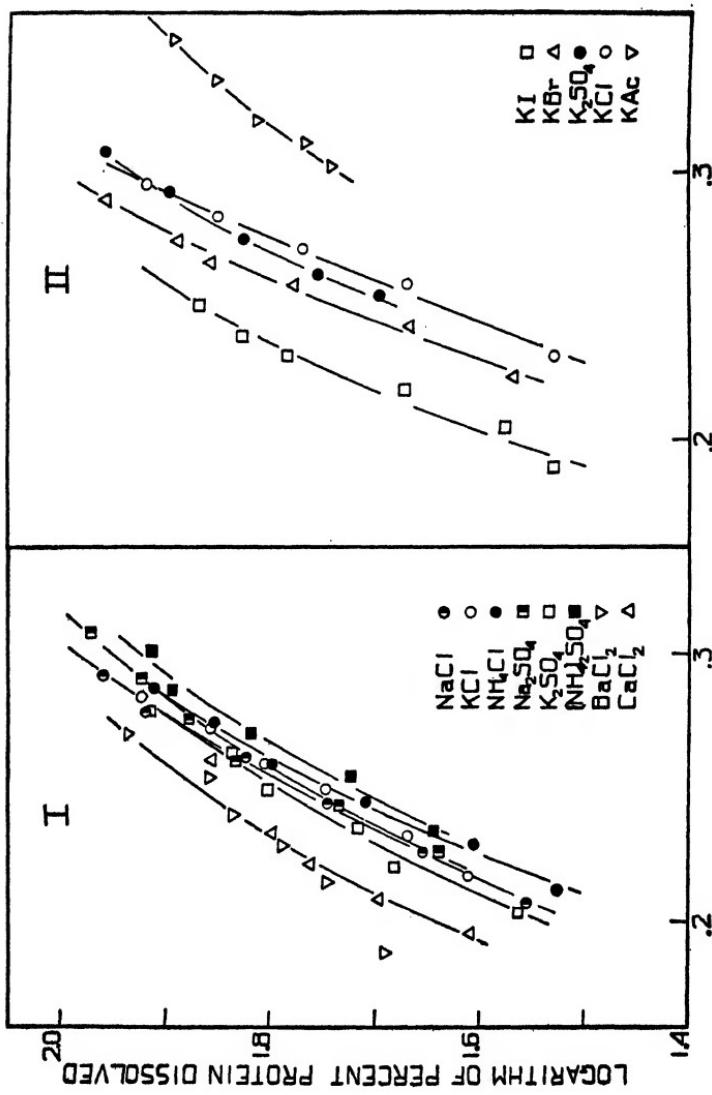


FIG. 3. The solubility of serum globulin in electrolytes. The points are calculated from the data of Mellanby (17).

may be described by the same curve as in  $\text{Na}_2\text{SO}_4$ , where the equivalent concentration of phosphate is greater than 0.5.

It is thus possible to describe the variation in solubility of hemoglobin with one constant characteristic of the protein in all electrolytes and one constant varying with the electrolyte.

The data may be accurately described, also, if one uses another theoretically unjustified expression, in which the equivalent concentration is substituted for the ionic strength in Equation 1. The values for  $K$ , and  $A$  are of course different and  $\log S_0$  is 1.30 and  $Z_1Z_2$  is again 4. The relation is, of course, again purely empirical, but is worth noting as a satisfactory means of describing the solubility of hemoglobin in electrolyte solutions. We refrain from giving constants and curves since the preceding equation is a simpler form and easier to use.

#### *Solubility of Serum Globulin in Solutions of Various Electrolytes*

Mellanby in 1905 (17) described the solubility of serum globulin in solutions of various electrolytes of different valence type. Some of his results are shown in Fig. 3, I. Since the total amount of globulin present affected the amount in solution he expressed his results in terms of per cent dissolved. This was done since the ratio of dissolved protein to total protein was found to be constant for any one concentration of salt. We have plotted the logarithm of the per cent dissolved which is analogous to the logarithm of the solubility used in the hemoglobin calculations. The electrolyte has been expressed as square root of ionic strength. It is evident that the curves for solubility are superimposable and that Mellanby was justified in deriving the "square of the valence" rule quoted above, since, of course, the ionic strength is a function of the square of the valence.

Mellanby also was not unaware of the divergence from this rule of ions of the same valence type. In Fig. 3, II we give his data for the solubility of serum globulin in various potassium salts. The decreasing order of the effectiveness as a solvent is  $\text{KI}$ ,  $\text{KBr}$ ,  $\text{KCl}$ ,  $\text{K}_2\text{SO}_4$ , and  $\text{KC}_2\text{H}_3\text{O}_2$ .

The range of electrolyte concentration investigated and also the range of solubility investigated makes it impossible to calculate

accurately the "apparent valence type" of the protein in terms of Equation 1. However, serum globulin "salts in" as though  $Z_1Z_2$  were about 12.

### *Solubility of Edestin in Solutions of Various Electrolytes*

Osborne and Harris studied the effect of many electrolytes on the solubility of the crystalline vegetable globulin, edestin. Some

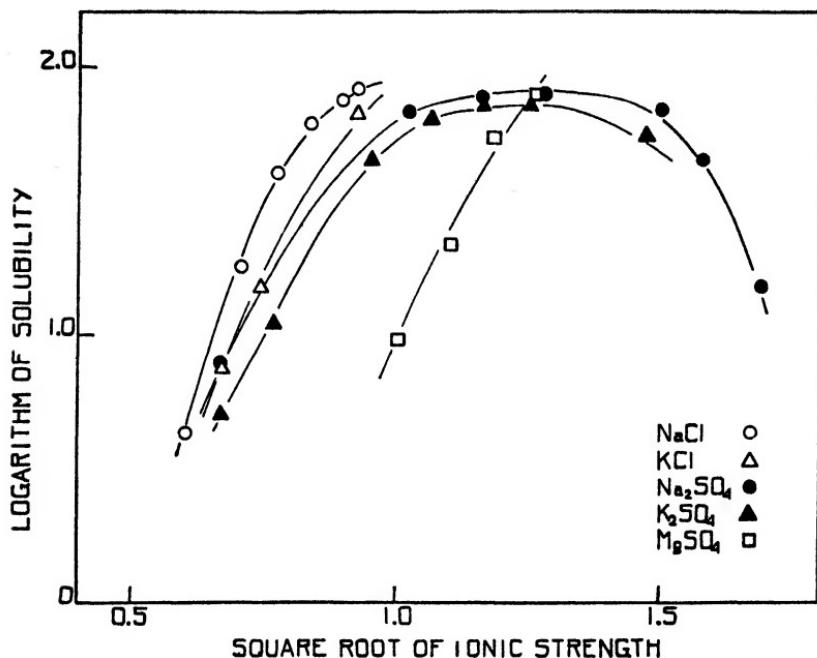


FIG. 4. The solubility of edestin in various salts taken from the data of Osborne and Harris (18).

of their data have been recalculated and are given in Fig. 4 in which we have plotted the logarithm of the solubility against the square root of the ionic strength,  $\frac{1}{2}$ . By comparing the solubility curves with those of hemoglobin in the same salts, it is evident that they differ in their steepness; that is, edestin has a much higher "apparent valence type,"  $Z_1Z_2$ , Equation 1. It is impossible to calculate the exact value, but  $Z_1Z_2$  is approximately 25 (6), whereas in

hemoglobin it is 4. In spite of this difference the variation in effect of the different salts at the same ionic strength is the same as in hemoglobin. Inspection of the curves shows that sodium chloride has a greater solvent power than potassium chloride. The chlorides have a greater solvent action than the sulfates. Magnesium sulfate has a lower solvent power in lower concentrations than sodium sulfate, but the protein is not readily salted out in magnesium sulfate and the sodium sulfate, on the other hand, is a very effective precipitating agent. All these effects of electrolytes on edestin are qualitatively the same as those of the same electrolytes upon hemoglobin.

Regardless of the form of equation used to describe the globulin-like behavior of hemoglobin in chlorides and sulfates the increase in solubility in dilute solutions of electrolytes as well as the precipitation in more concentrated solutions is thus qualitatively the same as that of other proteins in the same salts. Solubility is dependent upon temperature, upon pH, and upon the nature and concentration of the electrolyte employed. The magnitude of these various solubility phenomena is dependent upon the nature of the protein. The behavior of a protein in an electrolyte solution is dependent not only upon the electrolyte used, but also upon the character of the protein itself. Composition, available charge, hydration, molecular weight and "size," and probably many other factors must be taken into account before it will be possible to predict the behavior of any given protein in a given electrolyte solution.

I wish to thank Professor Edwin J. Cohn for his continued interest in these studies on the solubility of hemoglobin.

#### SUMMARY

1. The solubility of carboxyhemoglobin of the horse at approximately pH 6.6, has been measured in solutions of sodium and potassium chloride and of sodium, potassium, magnesium, and ammonium sulfate. Hemoglobin is a globulin whose solubility in sulfates exhibits a maximum with increasing salt concentrations.
2. The solvent power of dilute solutions of sulfates is much less than that of the chlorides.
3. The activity coefficients of the hemoglobin in these solutions

can be approximately described, but only approximately in terms of the simplified Debye-Hückel equation

$$-\log \gamma = \log S - \log S_0 = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A \sqrt{\mu}} - K_s \mu$$

where  $\log S_0$  is 1.20,  $Z_1 Z_2$  is 4,  $A$  is an empirical constant having approximately the same value as in other systems containing the same electrolytes, and  $K_s$  is also an empirical constant whose value is the same as that determined in concentrated solutions of electrolytes where the simple salting out expression holds.

4. The solubility of carboxyhemoglobin in chlorides and sulfates at 25° and pH 6.6 may be satisfactorily described in terms of the empirical equation

$$\log S = 1.30 + 1.6 \sqrt{C} - k_s C$$

where  $k_s$  is an empirical constant varying with the electrolyte.

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## A CHAMBER FOR MEASURING THE OXYGEN CONSUMPTION OF ANIMALS

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(Received for publication, November 9, 1931)

Several satisfactory chambers for indirect animal calorimetry are in use and the only purpose of this description is to present a more simple and practical type. The chamber as shown in Figs. 1 and 2 is built as an integral unit with the ventilating and absorbing systems self-contained.

The construction consists of  $\frac{3}{16}$  inch sheet steel with electrically welded joints. Supports are welded at intervals between the two outer walls which constitute the water chamber so that no bulging takes place from the water pressure. Pipe connections are also welded directly into the walls.

*Ventilation and CO<sub>2</sub> Absorption*—Adequate uniform air circulation without drafts is not so readily obtained when the air is pumped in and out of the chamber through pipe systems. In this design a large blower of the sirocco type is built directly into the end of the chamber so that no constrictions are placed anywhere in the air circuit. The air outlet at the top of the blower extends across the entire width of the chamber. The air passes over and through a layer of soda-lime a half inch deep supported on the screen tray extending the width and depth of the chamber. This arrangement permits the air to descend throughout the entire chamber without localized drafts and to pass through the lower screen supporting the animal from where it again enters the blower. The blower is sufficiently large so that it can run at a relatively slow speed (800 revolutions per minute). High speed blowers invariably have been found to be noisy. The motor is mounted on a rubber cushion and the stuffing box is also set in rubber gaskets.

If the soda-lime has the proper moisture content, the CO<sub>2</sub> is

practically completely absorbed and no correction has to be made for residual CO<sub>2</sub>. Table I shows the residual CO<sub>2</sub> in the chamber at the end of variable periods of time. The CO<sub>2</sub> content never was



FIG 1 Photograph of the chamber for measuring the oxygen consumption of animals

nil but it did not accumulate with time, so the soda-lime maintained a fairly uniform tension.

*Humidity*—It has usually been customary to use concentrated

sulfuric acid or anhydrous calcium chloride to take up the expired moisture. When soda-lime is used as a CO<sub>2</sub> absorber, these dehydrators are not practical because the soda-lime quickly becomes dry and loses its efficiency for absorbing CO<sub>2</sub>. Furthermore, animals are not comfortable over long periods of time when the air is too dry. In this design the principle has been used that the humidity remains constant in a confined space over a saturated

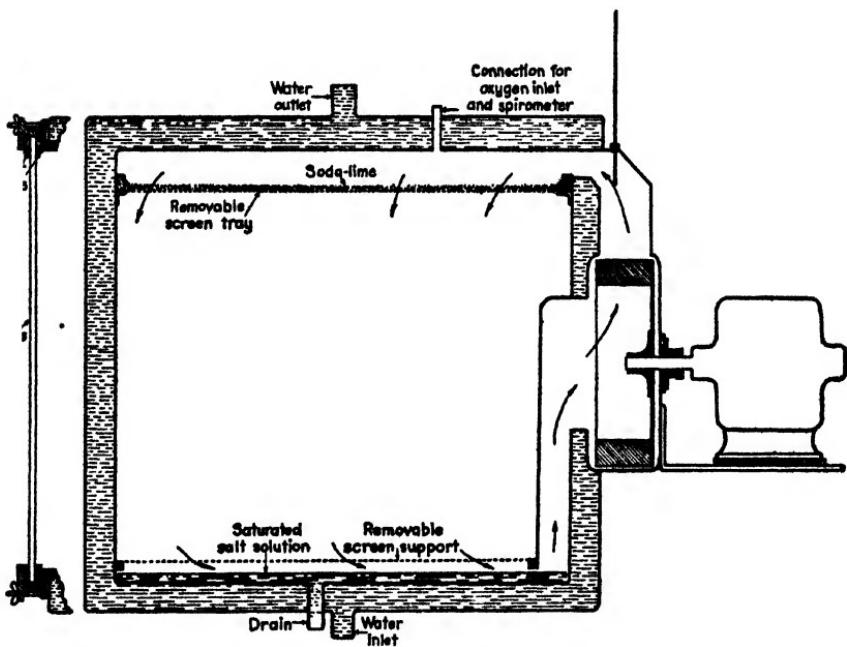


FIG. 2. Diagram of the chamber for measuring the oxygen consumption of animals. 1 inch on the figure represents 1 foot.

solution as long as an excess of the solid phase remains present and the temperature remains constant. By the choice of the solid phase, practically any desired humidity can be obtained. Table II, compiled from the literature, shows the range of humidity that can be obtained with some of the ordinary salts. The lower humidities are not practical when soda-lime is used and the values over 75 per cent are not conducive to quietness of the animal when the runs are of a prolonged nature.

**Temperature**—The maintenance of a constant temperature is obtained by means of the water jacket surrounding the chamber. This is connected at the top and bottom with a 100 gallon water tank in which the temperature of the water is kept constant by means of a thermoregulator and electric heater of the knife type. The water is circulated through the tank and chamber by means of

TABLE I  
*Residual CO<sub>2</sub> Content*

Observation No.	Time soda-lime was used	Run before analysis		CO <sub>2</sub> per cent
		hrs.	hrs.	
1	8		5	0.030
2	17		1	0.045
3	24		3	0.040
4	7		6	0.036
5	25		2	0.022
6	39		1	0.040
7	48		6	0.048
8	9		4	0.028
9	17		5	0.032

TABLE II  
*Constant Humidity*

Solid phase	t	Humidity	Aqueous tension	Bibliographic No.
	°C.	per cent	mm. Hg	
H <sub>2</sub> PO <sub>4</sub> ·½H <sub>2</sub> O.....	24.5	9	2.07	1
LiCl·H <sub>2</sub> O.....	20.0	15	2.63	1
CaCl <sub>2</sub> ·6H <sub>2</sub> O.....	24.5	31	7.15	1
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O.....	24.5	51	11.75	1
NH <sub>4</sub> NO <sub>3</sub> .....	28.0	61	17.30	2, 3
K <sub>2</sub> C <sub>4</sub> H <sub>4</sub> O <sub>6</sub> ·½H <sub>2</sub> O.....	28.0	73	20.70	4
NaCl.....	28.0	75	21.28	4, 5, 3, 6
KCl.....	28.0	84	23.83	4, 5, 2, 3, 6
K <sub>2</sub> SO <sub>4</sub> .....	28.0	97	27.50	4, 5, 1, 7

a fairly large direct drive centrifugal pump. 28° has been found the approximate optimal temperature for the best relaxation and consequently minimum oxygen consumption of dogs. When the external air temperature is warmer than 26°, it is necessary to run a small stream of cold water into the water system.

*Alcohol Checks*—Absolute alcohol was burned from a Mariotte constant pressure type of burette. Because of the downward circulation of the air in the chamber, some difficulty was obtained in having the alcohol flame burn without flickering. A deflector

TABLE III  
*Alcohol Combustion Values*

Experiment No.	Amount of alcohol burned cc.	Theoretical amount of O <sub>2</sub> required liters	O <sub>2</sub> consumed* liters	Deviation from theoretical per cent
1	3	3.460	3.495	+1.01
2	5	5.763	5.875	+1.95
3	4	4.619	4.577	-0.90
4	6.5	7.500	7.422	-1.04
5	5.2	6.000	5.970	-0.50

\* Reduced to 0°, 760 mm.

TABLE IV  
*Comparison of Various Methods of Calorimetry*

Dog 10, fasting 18 to 22 hrs.		Calories per 10 kilos per hr.*		
Date	Weight kg.	Chamber	Benedict	Tissot-Haldane
1930				
Jan. 10	18.20	13.5		
" 13	18.20	13.7		13.4
" 14		13.8		14.2
" 16		14.0		13.6
" 20	18.45	13.8		
" 21		12.8		
" 22		13.0		13.7
" 25		13.5	13.3	13.0
" 27	18.55	13.6	13.2	
" 28		13.6	14.6	
" 30	18.50		13.4	13.0
Feb. 3		13.3		
" 10	18.60	13.6	13.2	

\* Calorific value of 1 liter of oxygen is assumed to be 4.825.

was used over the lamp chimney and fairly uniform results were obtained. The results are tabulated in Table III.

*General Reliability for Dog Work*—The mechanical accuracy of this type of chamber is practically limited by the state of relaxa-

tion of the animal. The time required before a dog became accustomed to the chamber varied, of course, with different dogs. After a dog once was well trained, the results were quite accurate. Table IV shows the values obtained over a period of a month for one of our best trained dogs. This dog would curl up in the corner and apparently sleep during all of the runs. The table also shows the values that were obtained on the same dog with the use of a mask and the Benedict closed circuit type of apparatus, and also by collecting the expired air in a gasometer and determining the oxygen and CO<sub>2</sub> content. In this dog, which was well trained to relax, the values with the different methods agreed very well. In other dogs we found that it was much more difficult to obtain uniform values with the mask methods.

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## THE MEASUREMENT OF THE OXYGEN CONSUMPTION OF SMALL ANIMALS

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(Received for publication, November 9, 1931)

In the preceding paper Koehler describes an apparatus designed by him for measuring the oxygen consumption of dogs. His design has been modified by the writers to adapt it for use with small animals, such as mice, rats, and guinea pigs; mice and rats being the animals used in the test experiments.

The adaptation consists chiefly of a smaller animal chamber, a more exact method of controlling the temperature of the chamber, and a more sensitive method for measuring the amount of oxygen consumed. The chamber is a cubical box, 20 cm. to a side, about four or five walls of which circulates water from a large water reservoir kept at a temperature constant to  $\pm 0.005^\circ$ . A large De Khotinsky water bath is satisfactory for this purpose. The temperature of the chamber is determined by a Beckmann thermometer inserted through the top or side wall, and is considered constant when the variation is not more than  $\pm 0.005^\circ$ . In other respects this part of the apparatus is so like Koehler's as to require no further description.

The principal adaptation consists in the method of measuring the oxygen consumed. This may be done in two ways, either by a spirometer as illustrated in Fig. 1, or by a burette as illustrated in Fig. 2, the latter being the method used to obtain the results recorded in Tables I and II. The device for measuring the oxygen consumption by this method as illustrated on the right-hand side of Fig. 3 consists of a mercury receiving chamber communicating with the animal chamber and having attached to it a manometer, a burette, a stop-cock for admitting oxygen, and a

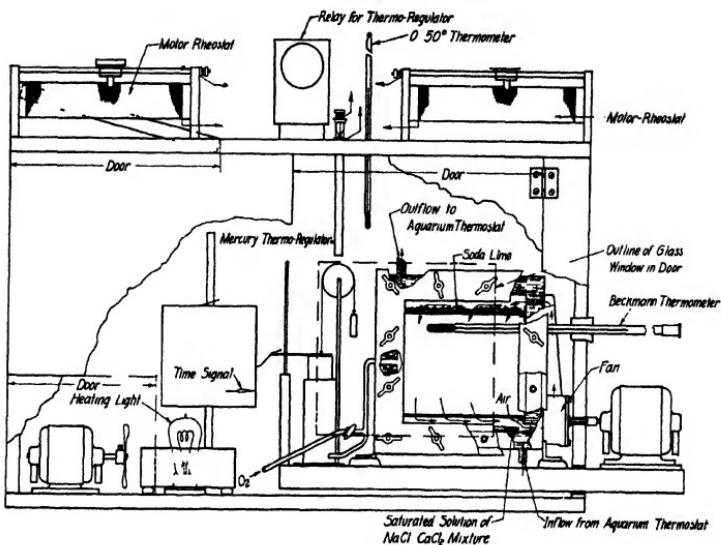
O<sub>2</sub> Consumption Measurement

FIG. 1. Metabolism chamber with spirometer attachment



FIG. 2. Metabolism apparatus with burette attachment

stop-cock for letting out mercury. This part of the apparatus is kept at the same temperature as the animal chamber by having both enclosed in an air bath whose temperature is regulated in the same way as that of the De Khotinsky water bath. The manometer is filled with kerosene and the burette with mercury. As oxygen is consumed by the animal the manometer levels change and

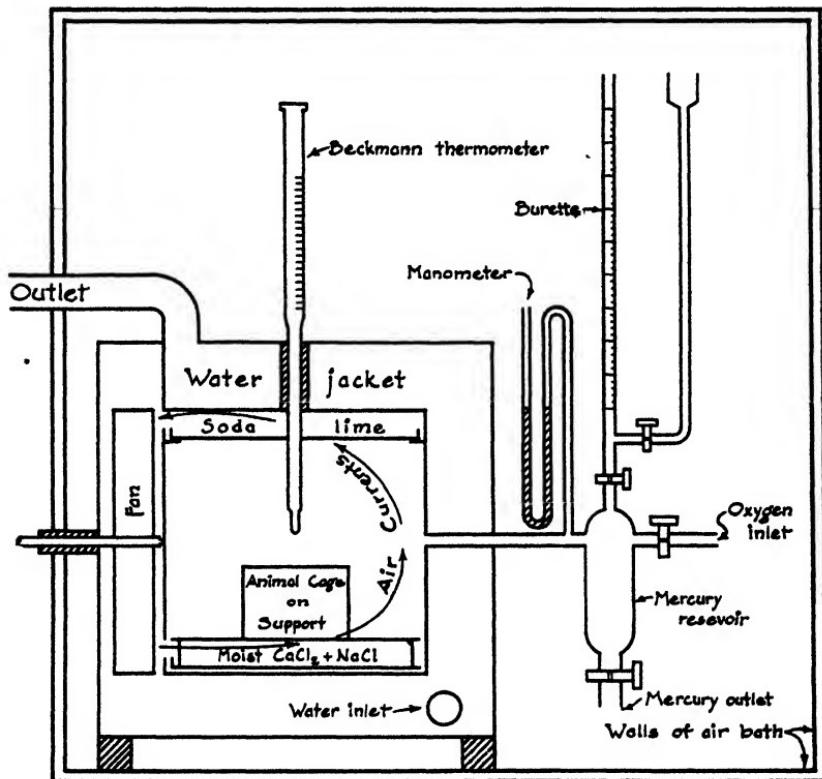


FIG. 3. Vertical section of metabolism chamber with burette attachment

mercury is run in from the burette to restore the manometer levels, the volume of the mercury run in being taken as a measure of the volume of oxygen consumed. As the barometric pressure usually does not vary over the period of a run which takes about 30 minutes, and, as the temperature of the animal chamber is kept constant at 28°, the volume of oxygen consumed can be deter-

**O<sub>2</sub> Consumption Measurement**

mined under conditions of standard temperature and pressure. It has been so expressed in liters of oxygen (S.T.P.) consumed per kilo of mouse per 24 hours.

The following results were obtained on mice that had become accustomed to experimental conditions, two or three preliminary

TABLE I  
*Oxygen Consumption of Mouse 4*

Time	State of mouse	Mercury
min.		cc.
0	Quiet awake	
5	" sleeping	2.9*
10	" "	2.0
15	" "	2.0
20	" "	2.2
25	" "	1.8
Total. . .		8.0
30	Quiet awake	2.5*
0	" "	
5	" relaxed	2.9
10	" "	2.8
15	" "	3.0
20	" "	2.9
Total. . .		11.6
25	Quiet sleeping	2.5*
30	" "	2.1
35	" "	1.9
40	" "	2.0
45	" "	2.0
Total. . .		8.0

Liters of O<sub>2</sub> (standard temperature and pressure) per kilo per 24 hrs. = 25.8 for quiet sleeping state and 37.4 for quiet relaxed state.

\* Not included in the total.

trials usually being necessary. The experiments were all performed about noon, 18 hours after the animal's last access to food. No readings were attempted until the mouse became quiet and the temperature constant. Trial readings were taken every 5 minutes, and, when these readings became approximately equal, the ex-

perimental period began and continued as long as the mouse remained in the same condition. The data for a typical experiment are recorded in Table I. The different values obtained for the different states of the mouse emphasize the importance of careful and continual observation of the mouse for which purpose the front of both the animal chamber and the air bath must be provided with glass doors.

59 such experiments as that recorded in Table I were performed on thirty-four normal, adult, white mice, 53 being for the quiet

TABLE II  
*Oxygen Consumption of Six Normal, Adult, White Mice*

Mouse No.	Date	Liters of O <sub>2</sub> (standard temperature and pressure) per kilo per 24 hrs.	
		Quiet sleeping state	Quiet relaxed state
	1931		
4	Mar. 19		37.5
4	" 21	25.8	37.4
8	" 10		38.2
8	" 11		37.6
8	" 12		37.8
8	" 15		38.9
14	" 14	27.8	37.0
14	" 16		37.6
14	May 13	28.1	37.5
18	Mar. 21		36.7
18	" 22	24.0	37.2
28	" 26	26.1	37.3
36	" 30	29.1	
Mean . . . . .		26.8	37.7
Probable error of mean . . . . .			0.72

relaxed state and six for the quiet sleeping state. The results of eighteen of these experiments including the latter six are shown in Table II. The 26.8 mean for the quiet sleeping state is based on too few cases to be considered reliable. It is, however, in very close agreement with the value obtained by Lee (1) on rats in a "quiet dozing" condition, the mean calculated from his data being approximately 27.0. The 37.7 mean for the quiet relaxed state is computed for the 53 experiments and the probable error, 0.72, indicates that the true mean lies between 37.7  $\pm$  2.88; that is,

between approximately 35 and 41. The often quoted value obtained by Rubner (2) for mice is 212 calories per kilo per 24 hours at 23–24°. Since our experiments were carried out at 28° a direct comparison of Rubner's figure and ours is not possible. However, if one applies Rubner's estimate of 2.5 per cent increase for every degree the temperature is lowered the figure for the metabolism of mice at 28° would be approximately 193 calories. Dividing this by 4.739, the factor given by Lusk (3) at R.Q. 0.75, for converting calories to liters of oxygen, one obtains 40.7 liters per kilo per 24 hours. This value is of the order found by our technique.

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## **PLASMA LIPIDS IN LACTATING AND NON-LACTATING ANIMALS\***

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Many studies have been made on the influence of the nature of the fat intake on the type of fat deposited in the reserves or secreted in the milk. Although the blood is the transportation system for the lipids, the few studies that have been made on the blood fat with respect to the ingested fat indicate that qualitatively and quantitatively they are not identical. Thus McClure and Huntsinger (12) have shown that human patients do not respond after duodenal administration and ingestion of single types of foodstuffs with corresponding types of blood fat. They concluded that a mobilization of tissue fats took place on the ingestion of all types of foodstuffs. Bodansky (3) also obtained similar findings recently with dogs fed on olive oil. He was unable to explain why, after the administration of olive oil, the increase in the total and saturated fatty acids, particularly in the corpuscles, should be greater than the increase in oleic acid.

In our study we undertook to obtain further information on the nature and distribution of the plasma lipids, particularly in relation to the fat intake and to lactation. Lactation provides an especially fine opportunity for studying the blood lipids, because a very intensive fat metabolism is occurring to provide the fat secreted in the milk and because the blood picture can be related not only to the food intake but also to the amount and character of the secreted product.

\* This manuscript is part of a dissertation presented to the Faculty of the Graduate School of Cornell University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. An abstract of this paper was reported to the American Chemical Society at the Buffalo meeting.

Cattle were selected as the experimental animals because large amounts of blood required were readily available. Six lactating and five dry cows as well as two steers were used. In four instances data were obtained on the same cow prior to and 1 month after parturition. This procedure allowed for fairly exact comparison between the lactating and non-lactating state.

#### EXPERIMENTAL

Approximately 2 liters of blood were obtained in oxalate from the jugular vein of dry cows and beef animals, while about 1 liter was taken from lactating cows 1 month after parturition. Plasma was separated and used in the following procedures.

*Total Fatty Acids*—The procedure used was a combination of Bloor's (2) alcohol-ether extraction and the Kumagawa saponifica-

TABLE I  
*Effect of Drying Oleic Acid at 120° and 28+ Inches Vacuum*

Period in min.	I.N.
0	90.1
5	89.7
10	88.8
15	88.1
20	85.0
25	83.3

tion method, as modified by Lemeland (8). Depending on the fatty acid content, 15 cc. or 25 cc. of plasma were used to determine total fatty acids and their iodine number.

After isolation the fatty acids were dried by placing in a desiccator overnight and heating *in vacuo* 5 minutes at 120°. They were cooled quickly on a cool, heavy metal plate in a desiccator, weighed, and their iodine number obtained by a micro-Hanus procedure. Table I shows the effect of heating *in vacuo* on the iodine number of oleic acid.

The 5 minute period, particularly after the fatty acid had stood in the desiccator overnight, was ample to dry the samples and this period was therefore adopted.

*Lipid Phosphorus*—5 cc. of plasma were precipitated in an alcohol-ether mixture and the lipid phosphorus determined by McCay's (11) modification of Denige's (6) method.

*Fatty Acid Distribution*—The procedure used was essentially that used by Bloor (1) and represented an approximate separation into phosphatides, cholesterol esters, and neutral fat.

About a liter of plasma in the case of non-lactating animals or  $\frac{1}{2}$  liter for lactating cows was precipitated in 2 volumes of alcohol and allowed to stand overnight. The precipitate was pressed and extracted with ether as suggested by Channon and Collinson (4). This was combined with the petroleum ether extract of the alcohol filtrate which had been distilled *in vacuo* at 45° to  $\frac{2}{3}$  its original volume. The ether was distilled off and the reddish yellow oily liquid dried in a desiccator.

The lecithin fraction was obtained by MacLean's (13) method. Cholesterol esters (Fractions I, II, and IV, Table V) were separated from the rest of the acetone-soluble lipid in alcohol by fractional separation in the cold. These were refractionated once from alcohol. After two fractions were obtained, Fraction III consisting of free fatty acids was removed by shaking an ether solution of the lipid with equal volumes of 0.1 N alcoholic KOH and water as was done by Bloor. After separation of the free fatty acids a further separation of cholesterol esters was made. This is designated as Fraction IV in Table V and was obtained in a similar manner as Fractions I and II. The final mother liquor (Fraction V) consisted chiefly of neutral fat and free cholesterol.

Aliquots of all fractions were saponified with sodium ethylate and the unsaponifiable material and the fatty acids recovered according to the Lemeland procedure. Iodine numbers were obtained on the fatty acids of the various fractions.

### Results

It was first felt desirable to see if there was any relationship between the time of feeding and the amount and nature of the fatty acids of the plasma as in the case of the carnivora. In Table II are given the data obtained on plasma taken at 2 hour intervals from a non-lactating cow fed at 5.15 a.m. and 1.15 p.m.

The concentration of total fatty acids was fairly constant throughout the day. This confirms the work of Porcher and Maynard (14) who attributed this to the large food capacity and the fairly continuous process of digestion in the cow. In addition we found a similar uniformity in the lipid phosphorus and in the

## Plasma Lipids

iodine numbers of the total fatty acids. This indicates that during the day there is a marked constancy in the nature as well as in the amount of plasma lipid of the cow and that the concentration of plasma lipids bears no relation to the time of feeding as in the carnivora.

However, the cows showed a noticeable individuality in the level of total fatty acids and lipid phosphorus under dietary conditions that were approximately the same. In Table III it is seen that

TABLE II  
*Effect of Time of Feeding upon Total Fatty Acids and Lipid Phosphorus*

Time	Total fatty acids mg. per 100 cc.	Iodine No.	Lipid phosphorus
			mg. per 100 cc.
5 a.m.	205	101	5.45
7 "	194	104	5.52
9 "	189	99	5.08
11 "	193	104	5.52
1 p.m.	204	98	5.28
3 "	198	97	5.52

TABLE III  
*Plasma Fatty Acids and Lipid Phosphorus in Lactating and Non-Lactating Cows*

The results are expressed in mg. per 100 cc.

Cow	Total fatty acids		Lipid phosphorus	
	Non-lactating	Lactating	Non-lactating	Lactating
A	148	297	4.09	6.45
H	156	320	4.71	7.19
G	163	365	4.78	7.14
K	184	409	5.03	7.30

the concentration of total fatty acids varied from 148 to 184 mg. per 100 cc. while the lipid phosphorus in the plasma of the same non-lactating cows varied from 4.09 to 5.03 mg. per 100 cc. A parallelism is thus noted between the total fatty acids and the lipid phosphorus in both the non-lactating and lactating state.

An interesting finding was that the cows having the highest total fatty acids and lipid phosphorus in their plasma in the non-lactating state also showed the highest values in the lactating state.

After 1 month of lactation the plasma total fatty acids and lipid phosphorus had risen to twice their former values. Average values on dry and lactating cows, as shown in Table IV, are similar to those recently published by Maynard, Harrison, and McCay (10) who followed these constituents during an entire lactation cycle.

Although the levels of the plasma lipids are considerably higher during lactation, the nature of the plasma fatty acids changes little if any. The iodine number of the total fatty acids of the plasma of lactating cows was slightly higher than that of non-lactating animals. It was felt that this might be accounted for by the relatively higher iodine number of the grain mixture which made up a larger percentage of the ration of the lactating cows. The plasma lipid values for steers were very similar to those for non-lactating cows.

TABLE IV  
*Plasma Lipids of Lactating and Non-Lactating Animals*

	Total fatty acids		Lipid phosphorus <i>mg. per 100 cc.</i>
	<i>Amount</i> <i>mg. per 100 cc.</i>	<i>I.N.</i>	
Non-lactating cows (5).....	168	101	4.70
Lactating cows (6).....	374	111	7.38
Steers (2).....	160	103	4.60

**Fatty Acid Distribution**—The data obtained in the approximate separation of phosphatide, cholesterol esters, and neutral fat are shown in Table V. The ether extraction of the protein precipitated with only 2 volumes of alcohol led to rather low yields of total lipid, due it is believed, to the imperfect penetration by ether of the large quantity of incompletely dehydrated plasma. When dehydrating large volumes of plasma to be followed by ether extraction of the protein, it is believed that better yields of lipids could be obtained if larger proportions of alcohol were used to dehydrate more thoroughly the plasma proteins. However, the lipid obtained is believed representative since different amounts of similar samples appear to approximate each other fairly well.

**Phosphatide Fraction**—As shown in Table V the fatty acids obtained from the acetone-insoluble fraction were of a lower degree

## Plasma Lipids

TABLE V  
*Distribution of Fatty Acids in Lactating and Non-Lactating Animals*

		Phosphate-free fractions from alcohol											
		Phosphatide fraction		Fraction I (ester)		Fraction II (ester)		Fraction III (free)		Fraction IV (ester)		Fraction V (fat)	
Sample		Amount	mg. per 100 cc.	I.N.	Fatty acids	I.N.	Fatty acids	I.N.	Fatty acids	I.N.	Fatty acids	I.N.	Fatty acids
Cow	cc.												
Lactating dairy cows	A	600	55.1	71	68.0	52.8	146	14.0	10.1	164	33.8	55	15.5
	G	630	91.3	53	26.3	22.8	33.1	22.0	13.5	24.0	33	15.3	10.3
	H	620	51.6	74	79.8	59.3	148	25.1	18.4	162	25.6	79	14.8
	I	485	108.0	57	69.3	54.4	151	48.5	36.3	144	34.2	39	17.1
	K	585	51.2	78	108.8	82.7	150	24.4	18.5	147	11.6	69	19.6
	N	600	56.5	76	88.6	63.8	152	54.0	38.8	146	7.8	91	25.6
Average.....		587	68.9	68	73.5	56.0	149	33.2	24.0	150	22.8	61	18.0
Non-lactating dairy cows	A	1150	6.3	100	44.1	56.0	130	18.5	14.6	148	2.0	89	2.3
	G	1120	22.8	62	35.2	37.1	111	17.2	14.7	148	3.8	99	5.5
	H	1010	7.6	83	34.0	36.1	106	20.6	18.5	132	3.1	85	1.2
	K	1160	12.4	82	69.6	45.4	118	6.2	5.2	147	14.0	79	6.6
	M	1150	3.6	100	38.4	39.0	111	7.6	5.8	153	1.1	84	6.3
Average.....		1118	10.5	85	44.3	42.7	115	14.0	11.8	146	4.8	87	4.4
Steers		1050	5.1	74	28.4	22.7	107	25.2	21.4	138	2.5	72	6.1
		1130	18.6	93	32.5	24.8	147	6.5	5.1	136	3.6	110	11.1

of unsaturation than those obtained from the other fractions. The average iodine number of the phosphatide fatty acids for the non-lactating group was slightly higher than for the lactating cows, due principally to two values somewhat higher than the others. No explanation can be given for this because feed and other conditions were similar for all the cows in this group.

The work of Levene and Simms (9) has shown that liver lecithin contains saturated and unsaturated fatty acids in equimolecular proportions. As Bloor has pointed out, if plasma lecithin is similar to liver lecithin, it would follow that the unsaturated fatty acids would have iodine numbers twice that found on the mixed acids from the phosphatide fraction. This would make the unsaturated fatty acids of the plasma phosphatide of both lactating and non-lactating animals similar in iodine number to that of the fatty acids combined with cholesterol esters.

The amounts of fatty acids obtained on saponification of the acetone-insoluble fraction were much lower than those calculated from the lipid phosphorus. LeBreton (7) has shown, however, that, in the case of tissues at least, the lipid phosphorus values may be as much as 20 per cent higher than the amounts obtained by direct isolation. Nevertheless, considerable decomposition of the phosphatide occurred during the process of isolation, as Bloor also found when using boiling alcohol as the extracting solvent of the protein. The large volumes of plasma required and the extended time involved during the process of isolation seem to be responsible for the lower yields of phosphatide. The iodine numbers of the free fatty acids (to be discussed later) which were undoubtedly derived from the decomposed phosphatide, were of the same order of magnitude as those obtained from the lecithin fraction, so it is believed that the iodine absorption values of the phosphatide fatty acids obtained are representative.

*Cholesterol Esters*—As has been stated, the cholesterol esters were separated on the basis of their lesser solubilities in alcohol than the neutral fats. They were reseparated once from alcohol and as obtained probably contained free cholesterol and neutral fat to a limited extent. They are reported under Fractions I, II, and IV in Table V.

A composite sample of the unsaponifiable material of Fractions II and IV showed by the Liebermann-Burchard color reaction that

they contained 90 per cent cholesterol. On the assumption that the proportion of cholesterol was the same in all the cholesterol ester fractions, calculations were made of the amount of fatty acid required if the cholesterol was present as cholesterol linolate, as suggested by Bloor (1). These calculated values in practically every case were somewhat lower than the fatty acids actually obtained. If consideration is given to the unknown but probably small amount of free cholesterol that is very likely admixed with the separated ester, the difference between the theoretical and actual values would be still greater. In addition the iodine numbers obtained on these acids were somewhat under that of cholesterol linolate. Channon and Collinson (5) working on whole blood were able to detect arachidonic, linoleic, and oleic acids in some of their fractions. From the ratio of cholesterol to fatty acid and the iodine numbers obtained, it seems likely that the blood plasma fatty acids in combination with cholesterol are mixtures—possibly of the three acids detected by Channon and Collinson.

The high degree of unsaturation of the acids of the cholesterol ester fraction might signify that they are taking some part in fat transportation. Although some evidence exists for this interpretation, the general chemical inertness of the esters and the lack of knowledge concerning the distribution and effectiveness of cholesterolase have led to an unwillingness to accept the cholesterol esters as participants in the metabolism of the fats.

The iodine number of the fatty acids of the grain mixture fed was 123 while that of the fatty acids of the hay was 65. Since the iodine number of the fatty acids combined with cholesterol was higher than that of the fatty acids in the food, it is clear that cholesterol exhibited selectivity in the type of fatty acid with which it combined. If it can combine with these highly unsaturated acids, it would seem reasonable to assume that cholesterol could combine with the unsaturated acids produced by the liver according to Leathes' hypothesis. In the blood plasma of lactating cows the actual quantity of *unsaturated* fatty acids combined with cholesterol as esters has been found to be greater than that in combination as lecithin. Qualitatively and quantitatively it would seem then, that these findings could best be interpreted as indicating a participation of cholesterol with lecithin in the trans-

portation of the highly unsaturated fatty acids during the intense fat metabolism of lactation.

The free fatty acids, as previously stated, undoubtedly have their origin in the decomposed phosphatide. Their iodine numbers are similar to those of the lecithin fraction and much lower than those obtained from the cholesterol esters and neutral fat. The amount of fatty acids obtained was also roughly proportional to the amount of phosphatide isolated, a finding which further points to their origin.

*Neutral Fat Fraction*—Fraction V was the mother liquor remaining from the separation of phosphatide and cholesterol esters. It was composed largely of neutral fat and free cholesterol. The iodine numbers of the fatty acids after saponification were approximately 100 while those reported on beef reserve fat averaged considerably below this value. Thus the circulatory neutral fat of these cows appears to reflect more nearly the weighted iodine number of the food fat than that of the reserves.

The writer wishes to express his thanks to Professor L. A. Maynard for his interest in this work and for his helpful advice and criticism.

#### SUMMARY

Total fatty acids, lipid phosphorus, and neutral fat were higher in the plasma of lactating than of non-lactating cows or steers.

There were individual variations in the levels of the various lipids in the plasma both in lactation and during the dry period. Animals which showed relatively high values when dry also gave high values during lactation.

In both lactating and non-lactating animals the plasma lecithin contained fatty acids of a much lower degree of unsaturation than those of the cholesterol esters, while the iodine number of the fatty acids of neutral fat was intermediate. The data suggest that the cholesterol esters may play a part in fat transportation.

Despite a marked change in the level of the various lipids from the dry to the lactating period, the character of the fatty acids, as measured by the iodine number, was similar. It seems evident, therefore, that lactation requires neither a difference in distribution nor any different kind of fatty acids, but merely a larger amount.

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## **INFLUENCE OF ANTERIOR PITUITARY SUBSTANCES ON THE TOTAL IODINE CONTENT OF THE THYROID GLAND IN THE YOUNG DUCK\***

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One of us has previously shown (Schockaert (1)) that injection of a saline suspension of fresh anterior pituitary glands induces in young ducks and guinea pigs a prompt and marked enlargement of the thyroid glands, reaching in two ducks 20 and 60 times respectively the weight of the controls. Histological study revealed that this treatment had induced changes indicative of a profound hyperactivity of the epithelium, colloid always having been excreted, the excretion in some cases being so extensive that in some vesicles no remnants of the previously abundant colloid could be distinguished. With continued treatment, colloid was again found but stained faintly and was granular in structure. All of these pictures presented a striking resemblance to those found in human thyroid glands in Graves' disease.

Quantitative determination of iodine in the thyroids of the experimental and control animals was thought to be of interest in order to add some information regarding the nature of the processes involved.

*Material*—The experimental material comprised twenty-five male ducks, all of the same race and hatching, which were 3 weeks old when the treatment was started. Detailed data about these animals will be found in the previously published paper (1). They

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were divided into six groups of three animals each, the groups being killed at intervals of 7 days. Each group consisted of one untreated control, a pituitary-treated animal, and a spleen-treated control. In addition three ducks were treated with a purified growth hormone (phyone,<sup>1</sup> van Dyke and Wallen-Lawrence (2)) and four with luteinizing hormones (three with a luteinizing hormone, Squibb, and one with prolan). They were killed after 1, 2, and 3 weeks of treatment with daily injections. The saline suspension of anterior lobe was injected daily at a dosage of 2 cc. for 500 gm. of recipient, corresponding to about 0.4 gm. of fresh tissue; *i.e.*, half of the anterior pituitary gland of an ox. The spleen emulsion used as a control was prepared according to the same procedure but was more concentrated, as the daily dosage corresponded to 1 to 1.2 gm. of fresh ox spleen.

The right thyroid lobes of all the ducks were analyzed for the total iodine, the left gland being used for histological study. In some cases, however, when the enlargement of the glands was particularly marked, a part of the left lobe was saved also for a control analysis. In these cases the findings from the two lobes were almost identical, results which show the reliability of the analyses.

*Iodine Determinations*—The iodine determinations were made by Leipert's (3) modification of Pregl's (4) method. The procedure is as follows:

The dried tissue is weighed into a platinum boat and burned in a stream of oxygen according to Pregl, the liberated iodine being retained in the cool end of the tube on glass beads moistened with sodium carbonate solution. The tube is washed out with about 40 cc. of water, the washings being collected in a 100 cc. Erlenmeyer flask. The solution is acidified to the turning point of methyl orange with 2 N sulfuric acid and then 1 drop of the acid is added in excess. The iodine is now oxidized to iodate by blowing bromine vapor<sup>2</sup> into the flask until the solution becomes pale yellow. The bromine vapor is delivered from a flask containing

<sup>1</sup> We thank Dr. van Dyke for kindly supplying the purified growth hormone (phyone).

<sup>2</sup> Some samples of bromine water developed a troublesome blank value on standing. The above expedient is convenient and entirely eliminates this difficulty.

saturated bromine water. The flask in which the bromine water is stored is provided with two sealed-in tubes; one to blow into, the other, drawn out to small bore and bent downward, for delivery of the bromine vapor. The neck of the flask is sealed off.

After addition of the bromine the solution is boiled down to a volume of about 8 to 10 cc. to expel the excess bromine. It is then cooled under the tap, about 20 mg. of potassium iodide crystals are added, and the solution titrated with 0.01 N thiosulfate from a micro burette graduated to 0.001 cc. Because of the oxidation of the iodine to iodate, 6 atoms of iodine are titrated for every 1 that was in the sample. Hence 0.01 cc. of 0.01 N thiosulfate is equivalent to  $2.12 \gamma$  of iodine from the sample ( $1 \gamma = 0.001$  mg.).

In working with such small amounts of iodine considerable care is necessary to exclude interfering substances, especially iodine and iron. The water used in reagents and for washing out the combustion tube should be redistilled from an all-glass still.

With pure reagents and the necessary care and cleanliness the blank on the method as a whole is zero and the starch-iodine color does not return within 5 minutes after completing the titration. When working with very small amounts of iodine (2 to 10  $\gamma$ ), the accuracy of the method is limited by the titration; the error in determining the end-point is not greater than  $\pm 0.001$  cc. of 0.01 N thiosulfate.

*Results*—The results of the determinations as detailed in Table I show a striking decrease appearing after pituitary treatment not only in the percentage of iodine present in the glands, which decreased 10 to 20 times, but also in the absolute amount of iodine. The total amount of iodine decreased as much after 1 week of treatment as after 5 weeks, but, since the glands greatly increased in weight, the percentage of iodine decreased more and more as the treatment went on.

While the three ducks which received the longest treatment (Ducks 78, 82, 74) showed the lowest iodine percentage, their thyroids showed much more colloid than that of the animal treated for the shortest time, in which case a higher percentage of iodine was present, while scarcely any colloid was left. Thus it seems that this pale colloid is chemically different from normal colloid and probably has different physiological properties. It may be recalled that 20 years ago Kocher (5) described a similar difference in the

TABLE I  
*Effect of Anterior Lobe Injections on Iodine Content of Thyroid Gland*

Duck No.	Treatment		Analysis			Fresh weight of both lobes per kilo body weight
	Substance injected	Days	Weight of dry right lobe	Total iodine in lobe	mg.	
88	None	7	4.64	13	0.28	65
70		14	6.27	18	0.29	77
					(0.27)*	
86		21	6.28	17	0.27	90
71		25	4.91	11	0.22	92
81		28	6.95	9	0.13	58
77		35	6.42	11	0.17	67
80	Spleen	7	2.62	12	0.46	69
75		14	4.92	10	0.20	88
					(0.21)	
73		21	6.00	11	0.18	65
84		25	1.76	4	0.23	47
76		28	3.15	5	0.16	75
85		35	12.63	12	0.10	109
83	Anterior hy- pophysis	7	12.03	2	0.017	251
72		14	13.38	3	0.022	223
					(0.023)	
87		21	8.41	2	0.024	137
					(0.015)	
78		25	37.74	5	0.013	550
82		28	33.70	5	0.015	360
74		35	55.07	3	0.005	1151
93	Growth hor- mone	7	7.38	5	0.07	159
79		14	6.79	2	0.03	100
91		21	15.56	4	0.03	193
					(0.03)	
90	Luteinizing hormone	7	2.84	7	0.25	67
89		14	4.31	9	0.21	73
					(0.27)	
92		21	10.27	5	0.05	120
99	Prolan	10	6.40	8	0.12 (0.12)	82

\* The figures in parentheses give the iodine percentage as checked in part of the left lobe.

iodine content of normal, deeply stained colloid and the pale colloid observed in human thyroids in Graves' disease.

The control animals, both spleen-injected and untreated, appeared to show a slight decrease in the percentage of iodine the longer they were kept, paralleling the slight histological changes described elsewhere (1). However, these slight changes are insignificant as compared with the effects of anterior pituitary injections.

The pituitary fraction containing the growth hormone seems almost as active in stimulating the thyroid as are the fresh suspensions, while the luteinizing hormone and prolactin, even with higher dosages, did not influence the percentage of iodine except in Duck 92, an unexplainable exception, as histologically no excretion nor hyperplasia had taken place.

Since the work of Oswald (6), Kocher (5), and chiefly Marine and his coworkers (7-9), it is well known that in man and animals the iodine content of the thyroid is inversely proportional to the degree of hyperplasia. Consequently, it appears from the data obtained in our experiments that the thyroid hypertrophy and hyperplasia induced with anterior pituitary suspensions can be compared, chemically as well as histologically, with the hyperplasia observed in human hyperthyroidism and exophthalmic goiter.

#### SUMMARY

1. The treatment of young male ducks with a fresh saline emulsion of beef anterior pituitary induced a rapid drop in the total iodine content of the thyroid gland.
2. The total iodine content is as low after a week as after 5 weeks of treatment (one-tenth to one-twentieth of the normal). The percentage of iodine continued to drop more and more since the glands undergo a marked hypertrophy and hyperplasia.
3. The anterior pituitary fraction which contains the growth hormone (van Dyke and Wallen-Lawrence's phyone) also possesses this thyrotropic action.

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## **THE CARBON DIOXIDE DISSOCIATION CURVE OF LIVING MAMMALIAN MUSCLE**

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The total amount of carbon dioxide in a cat or dog is somewhat greater than 1000 cc. per kilo, and is found principally in the blood, muscles, and bones. The blood contains about 25 cc. and the muscles about 125 cc. per kilo of body weight. The CO<sub>2</sub> content of blood and muscle is so easily variable as the situation of the acid-base equilibrium changes that a considerable amount of CO<sub>2</sub> may be given off from or retained by both blood and muscle.

The total amount of CO<sub>2</sub> in the muscles is significant when the ordinary rate of metabolism is considered. On the basis of a resting metabolic CO<sub>2</sub> production of 4 cc. per kilo per minute, it would require more than 30 minutes to produce the equivalent of the CO<sub>2</sub> contained in the muscles. Overventilation may reduce the muscle CO<sub>2</sub> content one-half, or ventilation with 10 per cent CO<sub>2</sub> may nearly double the normal content in a period of less than 2 hours.

The state of the CO<sub>2</sub> reserves in muscle may be partly inferred by frequent examination of the blood, but the blood is only a transporting medium in which a number of factors determine the amount of CO<sub>2</sub> transported. The only reliable method for ascertaining the integrity of the CO<sub>2</sub> reserve in muscle is direct analysis of the CO<sub>2</sub> content of several muscles before and after the period in question.

If the CO<sub>2</sub> tension in muscle can be estimated along with the CO<sub>2</sub> content, the principal variables are then known which characterize the acid-base equilibrium.

We have therefore attempted to determine the typical relation between CO<sub>2</sub> tension and content in living muscle of dogs, with the idea that it will be useful to indicate (1) the importance of

muscle CO<sub>2</sub> capacity in determinations of metabolism, and (2) the nature of the CO<sub>2</sub>-combining power curve and its derivatives.

The determination of CO<sub>2</sub> content and tension in living mammalian muscle offers certain difficulties inherent in the fact that the results must show the condition *in vivo* in tissues which are highly susceptible to injury. Both lactic acid and carbon dioxide are constantly being formed, and the rate of formation is likely to be disturbed by any experimental treatment of the muscles. The adaptation of appropriate analytical procedure and the necessary preparations and precautions of a physiological nature will be discussed after the general plan of the experimental method is outlined.

It is assumed that the CO<sub>2</sub> tension in the muscle can be determined from the tension in the venous blood coming from it. The CO<sub>2</sub> content of the muscle may be determined by analysis. These two quantities give one point on the curve relating CO<sub>2</sub> tension and content. A second point for the same animal may be obtained by examining the corresponding muscle of the other leg in the same manner. In order to secure points over a wide range of tension values, the animals may be overventilated or ventilated with a CO<sub>2</sub>-rich mixture after removing the first muscle. In this way two points for each animal are determined, and the slope of the dissociation curve is indicated for each individual.

#### *Methods*

*Total CO<sub>2</sub> Determination*—In the method of Ferguson and Irving (1929) muscle frozen in liquid air and analyzed by a modified Van Slyke manometric apparatus is used. Consistent results are obtained showing an average difference between right and left corresponding muscles of 2 per cent, or about 0.5 volume per cent with the ordinary content.

*CO<sub>2</sub> Tension in Muscle*—Theoretically the CO<sub>2</sub> tension in muscle must exceed that in the venous blood in order to maintain a gradient for outward diffusion. With the known diffusibility of CO<sub>2</sub> and the closeness of the capillary bed, Krogh ((1929) p. 273) figured that the difference in tension between muscle and venous blood would not be perceptible. We have consequently assumed that the venous blood CO<sub>2</sub> tension is identical with that in the muscle.

*CO<sub>2</sub> Tension in Venous Blood*—The usual methods for determination of CO<sub>2</sub> tension in blood require a larger volume than can be collected as venous outflow from a small muscle and are more or less indirect. For the purpose of these experiments, a method has been developed in which is used the principle of equilibrating a small air bubble with blood and analyzing the bubble. The blood flows directly into a pipette consisting of a capillary tube with a 2 cc. bulb. A small bubble of air is trapped with the blood and is small enough to have no perceptible effect on the existing blood equilibrium. The pipette is left in the water bath at 38° for equilibration and a small part of the bubble driven into the capillary tube for measurement. Next, the CO<sub>2</sub> is absorbed with alkali, and the measurement of the new volume shows the per cent of CO<sub>2</sub> in the air in equilibrium with the blood. An analysis of the accuracy of the method by two or three successive determinations on the same bubble shows a probable error of 2 mm. for each determination (72 determinations).

For sampling blood a long glass cannula is inserted through a branch into the main vein. Two samples of blood were taken from each muscle, and the probable discrepancy of the tension determinations on two successively drawn blood samples proved to be 3.4 mm. (twenty-four determinations). Consequently, it is shown that the CO<sub>2</sub> tension of the venous blood is practically constant under the experimental conditions for at least 5 minutes, which is greater than the interval between the removal of the last blood sample and the muscle for CO<sub>2</sub> content determination. The method will be described in detail in a separate publication (Ferguson, 1932).

*CO<sub>2</sub> Content of Blood*—Samples of venous blood and of carotid blood were also drawn directly into pipettes for CO<sub>2</sub> determination according to the method of Van Slyke and Neill (1924).

*Lactic Acid of Muscle*—The lactic acid content of pulverized frozen muscle was determined by an adaptation of the Clausen method, manganese sulfate being used as catalyst in the permanganate oxidation as proposed by Friedemann, Cotonio, and Shaffer (1927).

*Dry Substance*—The dry substance was determined gravimetrically after heating muscle samples at 107–110° to constant weight.

*Preparation*

The procedure was to isolate the venous return from the two gastrocnemii according to Verzár's plan (1912) and remove venous blood samples and one muscle. After a period of overventilation or ventilation with from 10 to 12 per cent CO<sub>2</sub>, venous blood samples were removed from the other gastrocnemius and the muscle itself removed. The whole treatment occupied about 5 hours and the success of the ventilation required inaction of voluntary control of the respiration. Since prolonged deep anesthesia and curarization are difficult to adjust and regulate, spinal dogs were used so that their movements and vascular adjustments would not be opposed to the unusual state of ventilation of the blood.

Dogs weighing between 5 and 7 kilos were dissected under ether anesthesia and the carotid and vertebral arteries tied. The spinal cord was then exposed and cut between the foramen magnum and first vertebra. Respiration ceased immediately; anesthetic was withdrawn and artificial ventilation maintained with the Schuster circulating respirometer.

Each gastrocnemius muscle was then exposed and all branches leading into the popliteal vein as high up as the upper limit of the popliteal space were tied off except those from the gastrocnemius and the small saphenous vein. The preparation to this point required from 1½ to 2 hours. The muscles were then covered with the skin and the animal allowed to rest and equalize its temperature for about an hour. During this period the rate of artificial ventilation was adjusted empirically to about 12 cc. per kilo per stroke and nineteen strokes per minute. Observations of the CO<sub>2</sub> content of arterial blood controlled the adequacy of the ventilation and were often available soon enough to suggest a small modification.

After the first resting period heparin was injected as an anti-coagulant. One muscle was then exposed and a long glass cannula inserted into the small saphenous vein at the level of the middle of the gastrocnemius, passing towards the popliteal vein. The pipette for tension determination was connected with the cannula and filled directly. Usually brief constriction of the popliteal vein was necessary to divert the blood into the pipette. After the last blood sample had been taken, the muscle was carefully dis-

sected, removed rapidly, and frozen in liquid air for determinations of CO<sub>2</sub> content, lactic acid, and dry substance.

The animal was next subjected to a modification of the method of ventilation which would assure a considerable change in tissue CO<sub>2</sub>. For overventilation the stroke volume and frequency of the pump could be increased to about 160 cc. per stroke and forty-eight strokes per minute. Various adjustments within this range were used, and while it was easy to overventilate, it was difficult to predict what degree of depletion of tissue CO<sub>2</sub> reserves would be produced. A frequent result of overventilation of these spinal animals is twitching of the skeletal muscles, which is often extensive but easily distinguishable from shivering. The overventilation reduces the alveolar CO<sub>2</sub> tension and consequently the CO<sub>2</sub> tension of the arterial blood. CO<sub>2</sub> is removed in larger quantities than it is produced by metabolism, and part of the CO<sub>2</sub> is removed from the muscles, which are therefore brought to a state of lower CO<sub>2</sub> tension. The degree of overventilation may be indicated by the elevation of the respiratory quotient above the normal level. It may be that with moderate overventilation a new state of equilibrium would be reached in which vascular compensation prevented further removal of CO<sub>2</sub> and the animal would return to a steady state with all of its tissues at a CO<sub>2</sub> tension lower than normal. But the attainment of such a state of equilibrium was not often observed in previous experiments (Irving, Ferguson, and Plewes, 1930). Consequently the overventilation was carried on for an arbitrary time lasting from 1 to 2 hours, at the end of which it could be shown that the muscle CO<sub>2</sub> tension did not change within a period of about 10 minutes.

In order to raise the CO<sub>2</sub> tension in the muscles the pump inlet was connected with a reservoir of oxygen containing from 6 to 12 per cent of CO<sub>2</sub>. Under such conditions the elimination of CO<sub>2</sub> is hindered and CO<sub>2</sub> accumulates in the body. A considerable part is retained in the muscles (Irving, Ferguson, and Plewes, 1930) but in contrast to overventilation experiments, saturation is reached within 1 or 2 hours and the respiratory quotient returns to the normal value (Shaw, 1926). Consequently, a true state of equilibrium may be reached, with the muscles at a CO<sub>2</sub> tension higher than normal.

After the CO<sub>2</sub> tension of the muscles had been altered, blood

samples were again drawn and the muscles removed and fixed in liquid air for determination of CO<sub>2</sub> content, lactic acid, and dry substance. The temperature of the animal was observed with a thermometer inserted into the tissues under the axilla, and the temperature of the gastrocnemius was observed by means of a thermometer in the popliteal space. These muscle temperatures were between 36 and 39°, and were maintained by the use of a heated table and blankets.

#### *Relation between CO<sub>2</sub> Capacity and Tension*

The results of observations on thirty-five dogs are shown in Table I. In twenty-one of these, CO<sub>2</sub> content and tension were determined in both gastrocnemii. Of the other fourteen, in five cases the animals were prepared for other experiments on the second muscle, and in the remaining nine either the dog failed to survive or some accident prevented completion of the analyses. No experiments are omitted, for the criterion of survival with adequate blood flow and proper temperature precluded the chances of obtaining results on grossly abnormal animals.

The question now arises as to what curve best fits the points for CO<sub>2</sub> content and tension. In making a selection, two considerations require attention. First, the curve must be defined exactly, so that it may be reproduced accurately for purposes of comparison or calculation. Secondly, the shape and slope of the curve must not lead to factitious conclusions when its derivatives—the CO<sub>2</sub> dissociation curve, and more particularly the buffer power—are being considered.

It is obvious from the scatter of the data that considerable difference of opinion is possible as to what form of curve would give the fairest representation of the experimental points and at the same time fulfil the first requirement. Because of the great effect the slope and shape of such a curve have on the conclusions which might be drawn about the buffer power of muscle, four types of curve have been fitted to the data, and all of them, together with their derivatives, must be given due weight in formulating any general conclusions. They are all shown on one graph in Fig. 1.

*Curve I* (Fig. 1)—From  $p\text{CO}_2 = 45$  mm. of Hg to  $p\text{CO}_2 = 200$  mm. of Hg the points may be represented by a straight line equation, total CO<sub>2</sub> (in cc. per 100 gm.) = 19 + 0.151 ( $p\text{CO}_2$ ). From

TABLE I  
*Changes in Dog Muscle Following Overventilation or Ventilation with  
 10 to 12 Per Cent CO<sub>2</sub> in Oxygen*

The second line in each experiment is for the second muscle, examined 1½ to 3 hours after the first.

Experiment No.	Muscle			Total CO <sub>2</sub>			Experiment No.	Muscle			Total CO <sub>2</sub>		
	Total CO <sub>2</sub>	Dry substance	Lactic acid	pCO <sub>2</sub>	Venous blood	Arterial blood		Total CO <sub>2</sub>	Dry substance	Lactic acid	pCO <sub>2</sub>	Venous blood	Arterial blood
	cc. per 100 gm.	gm. per 100 gm.	mg. per 100 gm.	mm. Hg	cc. per 100 gm.	cc. per 100 gm.	cc. per 100 gm.	gm.	mg. per 100 gm.	mm. Hg	cc. per 100 gm.	cc. per 100 gm.	
1 30.4	48.4	16	33.6								88	54.0	
21.5	61		13.2	17	23.9	25.8	0.23	48	26.0				
2 26.2	22.0	61	41.5		36.4	25.8	0.15	112	38.0				
18.8	23.5		26.6	18	31.2			94	34.3				
3 27.5	25.6	0.18	53	42.2	41.9	48.2				186	50.0		
18.5	25.8	0.21	37	34.4	29.4	19	32.2	26.8	0.35	62	27.5		
4 29.4	22.8	0.33		44.5	41.2			25.4	0.50			39.0	
17.6	23.9	0.43	27	30.4	28.2	20	23.3	26.5	0.25	56	25.5		
5 25.7	24.8	0.27	45	47.6	41.5	28.5	29.5	0.24	105	27.0			
15.4	24.3	0.38	38	31.3	27.9	21	26.4		0.20	55	27.0		
6 27.7	26.2	0.27	49	42.6	35.0	30.2		0.32	75	30.5			
20.0	26.6	0.53	34	29.6	16.3	22	30.7	25.0		51	24.8		
7 26.5	0.32			45.8	43.0	32.4	25.0			68	26.5		
19.5	0.50	32.5	30.9	20.8	23	28.4		0.14			40.6		
8 28.0	22.2	0.19	57.0	50.0	47.0	20.0		0.26			29.6		
16.7	22.5	0.35	42	31.3	20.2	24	32.0			66	46.5		
9 24.2	0.37	53	39.3	40.0	25	35.3	29.0	0.33	108	45.6			
13.6	0.77	40	24.0	18.7		16.3	27.0	0.23	41	28.4			
10 32.6	0.95	125	49.0	40.8	26	31.1	26.2	0.48	76	49.2			
44.3	0.70	203	63.0	59.8		17.0	26.4	0.44	20	25.4			
11 29.2	0.40			41.6	27	37.7				90			
38.6	0.34			48.5	28	35.8	24.6	0.68	96				
12 36.7	0.51	118		61		16.3	24.7	0.27	49				
46.0	0.41	166	67.0	62.4	29	29.5	32.1	0.60	64				
13 33.2	0.58	100	56.0	40.0	30	32.0		0.41	75				
46.1	0.52	181	70.3	69.5	31	28.2	34.5	0.72	72				
14 26.7	0.33	86	43.8	44.2	32	33.7				85			
35.7	0.32	98	54.0	47.1		25.8	25.4	0.37	54				
15 33.4	0.58	97	50.0		33	27.3				68			
42.2	0.72	137	53.2			45.4				170			

the end of this line at  $p\text{CO}_2 = 45 \text{ mm.}$  a straight line or a free-hand curve may be drawn to the origin. To the eye this curve represents very well the range above  $p\text{CO}_2 = 45 \text{ mm.}$  but gives little weight to the points at lower tensions than  $p\text{CO}_2 = 45,$  which are for the most part below the curve. When buffer power is studied by plotting a curve of  $\text{BHCO}_3$  against pH (Fig. 3), a tremendous

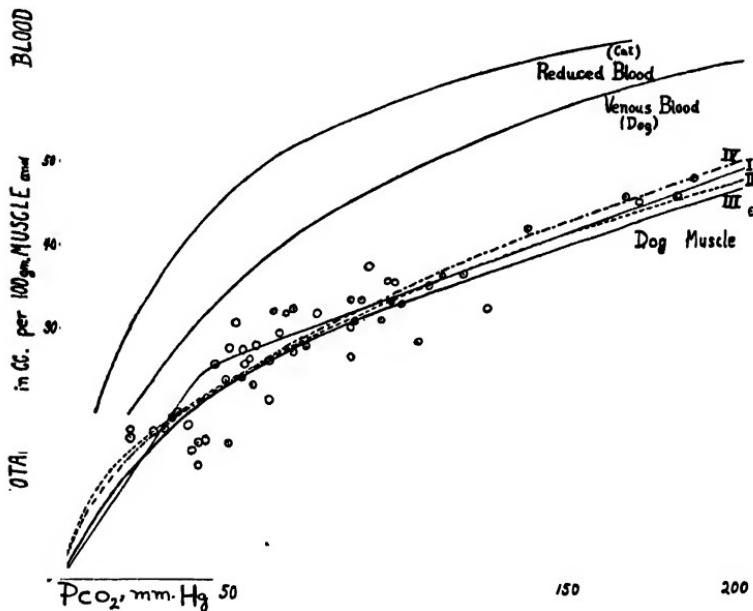


FIG. 1. Experimental results are indicated by circles. The curves fitted to them (Curves I to IV) are shown for comparison. The derivations of these curves are discussed in the text. For the venous blood of the same dogs an aggregate curve is shown, but the data themselves are not. Though of questionable value for purposes of comparison, the reduced blood curve of a single cat is included.

and improbably sudden inflection in the curve is apparent. From pH 7.04, with increasing pH, the buffer power rapidly approaches infinity as a consequence of the more or less straight course Curve I pursues to its origin. Just above pH 7.04 the buffer power is very small, but as a consequence of the straight course of Curve I above  $p\text{CO}_2 = 45 \text{ mm.}$  the buffer power again rises rapidly towards infinity with decreasing pH. It is hard to imagine that the

conditions represented at the ends of this curve are anything but factitious. Over the middle ranges, from  $pCO_2 = 60$  to  $pCO_2 = 110$ , the curve seems to fit the data well, and its derivatives should be fairly reliable.

*Curve II (Fig. 1)*—This curve is defined by the equation, total  $CO_2$  (in cc. per 100 gm.) =  $3.4 \times \sqrt{pCO_2}$ . This form has been found to represent adequately the results of Stella (1929) for frog muscle up to tensions of 200 mm. We have fitted it to our data by plotting  $[CO_2]$  against  $\sqrt{pCO_2}$  and finding the best straight line passing through the points and the origin. Its slope is 3.4. When transferred to the scale of Fig. 3, this curve appears to the eye to be higher than the data would warrant in the lower ranges of  $pCO_2$  and lower than they would warrant in the highest ranges. In the middle range it does not appear to give quite enough weight to a considerable number of high points between  $pCO_2 = 50$  and  $pCO_2 = 75$ . The buffer power shows at first a small, gradual rise with decreasing pH and later a slight fall.

*Curve III (Fig. 1)*—Curve III is drawn smoothly through the middle of the area covered by the points. This area is delimited above and below by two curves, drawn free-hand to skirt the points. To the eye this curve expresses the data very satisfactorily (with, perhaps, the exception of a group of high points between  $pCO_2 = 50$  and  $pCO_2 = 75$ ) but does not fulfil rigidly the first requirement. Derived from this curve, the buffer powers show an increase at both ends of the range, but the changes are not nearly so accentuated, or sudden, as in those derived from Curve I.

*Curve IV (Fig. 1)*—This curve approximates the  $CO_2$  dissociation curve for blood and is defined by the equation, total  $CO_2$  (in cc. per 100 gm.) =  $2.87 (pCO_2)^{0.542}$ . The curve was fitted to the data by plotting  $\log CO_2$  against  $\log pCO_2$ . The best straight line passing through the points was chosen. Its equation  $y = 0.458x + 0.542$  fixes Curve IV ( $y = \log CO_2$  and  $x = \log pCO_2$ ). To the eye this curve appears to be too high in the extreme upper and lower ranges. The buffer power tends to rise with decreasing pH but not as much as when derived from Curves I and III.

Curve III fits the data well, but is not reproducible, and while Curve I fits well, its derivatives indicate its unsuitability. Curve II fits somewhat better than Curve IV, and because it is easily defined and gives a reasonable appearance to the derivatives (to

be discussed later), it will be taken as the best summary of the data.

The lower limit of these curves is theoretically the point of  $p\text{CO}_2 = 0$  and CO<sub>2</sub> content = 0. The lowest tension reached was 20 mm., and the points for tensions between 20 and 45 mm. are predominantly below any curve which is drawn to reach the origin. These lower points were all obtained after from 1 to 3 hours severe overventilation, so that there was an opportunity for the CO<sub>2</sub> capacity of the muscle to change during the interval between removal of the first and second muscles. It is natural to suspect that lactic acid accumulates during the overventilation as was suggested by Macleod and Knapp (1918). In the nine complete experiments on overventilated dogs the average increase in lactic acid was 0.12 mg. per gm., which would elevate the curve by the equivalent of 3 cc. of CO<sub>2</sub> along an iso pH line. A correction of this size would raise the average curve (Curve III) in the overventilated cases (below  $p\text{CO}_2 = 45$ ) to a position between Curves II and IV and indicates that they more closely represent the conditions in unchanged muscle.

The only other factor which might cause a change in CO<sub>2</sub> capacity after overventilation that was investigated was the water content of the muscle, which is known to increase considerably during exercise. There was no significant change.

If the amount of lactic acid formation suggests that the true curve is above the average curve for tensions less than 45 mm., then lactic acid should be considered in evaluating the probable correctness of the curve for tensions between 45 and 200 mm. From the figures in Table I it is noticeable that the lactic acid content of the muscles diminishes in seven out of nine determinations made after ventilation with 6 to 12 per cent CO<sub>2</sub> in oxygen. The decrease in lactic acid may be due to improved oxygenation, to dilatation of muscle capillaries from the increased acidity, or it may be that the acidity itself depresses the formation of lactic acid. The cause is incidental to the main argument, which is to consider whether the decrease in lactic acid has significantly increased the CO<sub>2</sub> capacity.

The average change in lactic acid for the muscles at high CO<sub>2</sub> tensions was found to be 0.035 mg. per gm. of increase. This would lower the upper extremity of the curve by about 0.5 cc. of

$\text{CO}_2$ , and by this amount it would increase the distance between Curve III and Curve II if the correction were applied to the former.

An important piece of evidence for the correctness of the curves is obtained from an examination of the  $\text{CO}_2$  content-tension curve for the venous blood from the muscles examined if we employ the same tension figures as were used for muscle  $\text{CO}_2$  tension. The shape of the curve for reduced blood is well known and is indicated as the average for several samples of cat blood in Fig. 1. The curve for dog venous blood differs from that for cat reduced blood in several respects. Some of these differences can be attributed to known influences which would operate in experiments of this sort, and which, when accounted for, indicate that the venous blood is about normal. In the first place, the venous blood curve is lower than that for reduced blood, a change which can be attributed partly to dilution following hemorrhage during the prolonged experimental treatment of the animals, and partly to the fact that the oxygen content of the venous blood was greater, varying between about 4 and 15 volumes per cent. A curve for partly oxygenated blood is lower than that for the same blood when fully reduced. When these factors have been allowed for, the average curve for the venous blood of the dogs examined approximates the typical reduced blood curve, and shows that the assumption of stability in the acid-base equilibrium during the experiments is substantially correct.

#### *Combined $\text{CO}_2$*

The Curve II defined in the preceding section expresses the relation between  $\text{CO}_2$  content and tension of dog muscles with reasonable accuracy. From these data the  $\text{CO}_2$  dissociation curve may be calculated to show the relation between  $\text{CO}_2$  tension and the amount of combined  $\text{CO}_2$ .

The amount of the total  $\text{CO}_2$ , determined is the sum of the dissolved  $\text{CO}_2$ , represented as  $\text{H}_2\text{CO}_3$ , and the  $\text{CO}_2$  combined with base as bicarbonate. In aqueous solution the amount of  $\text{H}_2\text{CO}_3$  can be calculated from the expression

$$\text{H}_2\text{CO}_3 \text{ (in cc. } \text{CO}_2 \text{ per cc. solution)} = \frac{P}{760} \alpha$$

where  $\alpha$  is the Bunsen solubility coefficient and  $p$  is partial pressure of CO<sub>2</sub> in mm. of Hg. The water content of twenty-seven dog muscles examined averaged 74.7 gm. per 100 gm. of muscle, which is not an ordinary dilute solution. However, dissolved substances in muscle affect the vapor tension and apparently osmotic pressure of muscle about as they would a true solution in the same volume of water as is contained in the muscle, so that practically the whole amount of water may be considered as free (Hill, 1930). There are, nevertheless, at least two systems of solutions present, the muscle plasma and the cells, which are osmotically equivalent but are not necessarily equivalent in the concentration of each dissolved constituent. There is no information at hand to allow for discrimination between the two systems of solutions, so that for the purpose of calculation it must be recognized that the figures for H<sub>2</sub>CO<sub>3</sub> and BHCO<sub>3</sub> represent the net result and may be false for each system alone. However, the estimation of the net result is necessary for the subsequent application of the Henderson-Hasselbalch equation.

If the CO<sub>2</sub> is regarded as dissolved in 75 per cent of the muscle's weight of water, the solubility may be influenced by the factors which are known to modify the solubility of CO<sub>2</sub> in blood plasma and cells (Van Slyke, Sendroy, Hastings, and Neill, 1928). It was found that about 1 per cent more CO<sub>2</sub> can be dissolved in plasma than in the amount of pure water which the plasma contains. The cell water apparently has its CO<sub>2</sub> solubility increased by about 10 per cent. Muscle has about the same water content as blood cells, so that there may also be an increase in the CO<sub>2</sub> solubility. But since there is no direct evidence, it is better to assume that the CO<sub>2</sub> dissolved in muscle is that which would be dissolved in the muscle water. That makes the value of  $\alpha$  gm. (muscle) at 38° = 0.75 ×  $\alpha$  (water) at 38°, or 0.41, where  $\alpha$  gm. = cc. of CO<sub>2</sub> dissolved in 1 gm. of muscle when the partial pressure of CO<sub>2</sub> is 760 mm. of Hg.

The CO<sub>2</sub> combined has been calculated by subtracting the dissolved CO<sub>2</sub> from the total for each curve in Fig. 1. It is represented graphically in Fig. 2, showing the relation between CO<sub>2</sub> tension and combined CO<sub>2</sub>. The curves for frog muscle obtained by Fenn (1928) and Stella (1929) are also shown, but since they were made at room temperature they are not comparable with the curve

for dog muscle at 38°. At 38° they would probably be much lower, but it is difficult to estimate a temperature correction.

The curve showing total CO<sub>2</sub> capacity of muscle has been compared with that for venous and reduced blood in Fig. 1. It is evident that if Fenn's curve were fitted to the terms total CO<sub>2</sub>,

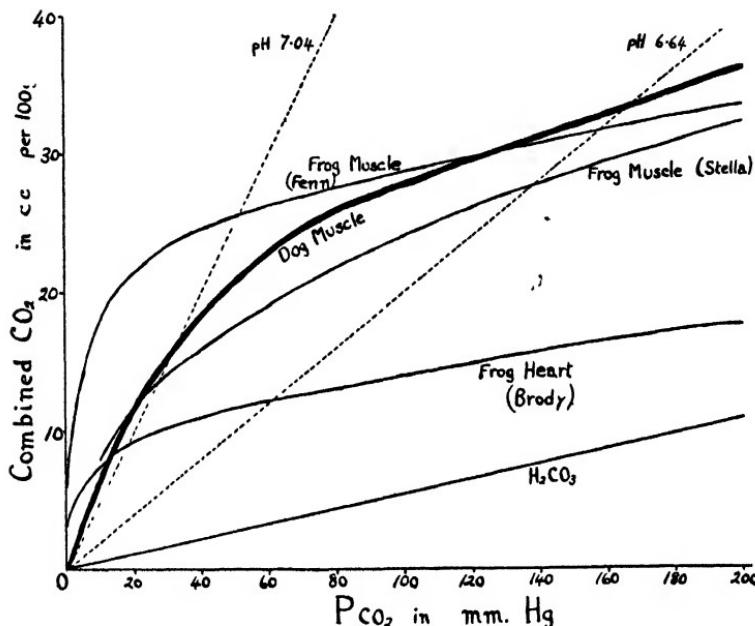


FIG. 2. The combined CO<sub>2</sub> in dog muscle (*in vivo*) at each CO<sub>2</sub> tension has been obtained by subtracting an estimated figure for the free CO<sub>2</sub> from the total CO<sub>2</sub> shown on Curve II, Fig. 1. The free CO<sub>2</sub> (H<sub>2</sub>CO<sub>3</sub>) at each tension is represented by the lowest curve.  $\alpha$  gm. at 38° is assumed to be 0.41 cc. of CO<sub>2</sub> per gm. of muscle. pH lines for dog muscle at 38° are shown, calculated on the further assumption that pK'<sub>1</sub> = 6.07. The CO<sub>2</sub>-combining power curves recorded by other workers are included. In the experiments of Fenn the temperature was 22°; in Brody's 22° (?). In the experiments of Stella the temperature was 18°; his data are uncorrected for lactic acid.

and tension, it would have approximately the same shape as the blood curve, while our curve for mammalian muscle and Stella's are different, rising more gradually at tensions below 50 mm. and subsequently rising more rapidly than the blood curve, although at a lower level.

*Application of Henderson-Hasselbalch Equation*

The data of the CO<sub>2</sub> dissociation curve may be used in the Henderson-Hasselbalch equation for the calculation of pH of muscle. pK'<sub>1</sub> has been given the value 6.07, as used for serum by Van Slyke *et al.* (1928). The results are shown on the dissociation curve (Fig. 2) by lines intersecting the pH points, as suggested by Haggard and Henderson (1919), and in Fig. 3 for convenience in the later calculation of buffer values. The pH values can be compared with estimations of other workers by consulting Table II.

TABLE II  
pH of Resting Muscle

Animal	Method	Temper- ature °C.	pH	Observer
Frog	Electrometric; juice	Room	7.23	Pechstein (1915)
	" aqueous extract	?	7.1-7.3	Meyerhof and Lohmann (1926)
	Calculated from CO <sub>2</sub> -combining power	22	7.2-7.3	Fenn (1928)
Mammal	" "	17-19	6.98	Stella (1929)
	Electrometric; aqueous extract	16-20	6.05	Michaelis and Kramsztyk (1914)
	Needle electrode	38	7.2	Schade, Neukirch, and Halpert (1921)
Cat	Glass " juice	38	7.15	Furusawa and Kerridge (1927).
	Quinhydrone; aqueous extract	25	7.4	Mackler, Olmsted, and Simpson (1930)

The results of electrometric determinations do not show the CO<sub>2</sub> tensions at which the determinations were made, and several workers report that the loss of CO<sub>2</sub> apparently had little effect. The effect of CO<sub>2</sub> loss may be small when the pH is greater than 7, but for the lower values it is certainly considerable. Most of the results indicate that the pH is less than that of blood, in spite of the probable loss of CO<sub>2</sub>. Our results indicate that at CO<sub>2</sub> tensions between 50 and 60, which probably prevail in resting muscle, the pH would be between 7.0 and 6.9. It follows from the fact that the bicarbonate of muscle is not much more than half

that of blood, that the muscle must always be more acid, unless the values of  $\alpha$  and  $pK'_1$  are unexpectedly different.

Here it may be noted that a pH calculated from such data does not represent the true pH of any phase of the muscle. If a Donnan equilibrium exists between these phases, the calculated or aggregate pH will differ from the true pH of any phase by an added constant and will be intermediate to the true pH of the various phases. For the determination of buffer power change in pH is the essential point to be considered, and for this reason, if for no other, the calculation of an aggregate pH is justifiable.

### *Buffering Power of Muscle*

The buffering power ( $\beta$ ) is defined by Van Slyke (1922) as the ratio of change in base to change in pH, or  $\beta = \frac{\Delta B}{\Delta pH}$ , in which

$-\Delta B$  = gm. equivalents of strong acid added to each liter of the solution. In dealing with muscle we have let  $-\Delta B$  = gm. equivalents of strong acid per kilo of muscle. In the buffering system of muscle we can distinguish two components, (1)  $H_2CO_3$  and  $BHCO_3^-$  and (2)  $Ha$  and  $Ba$  where "a" indicates all of the weak acid anions beside  $HCO_3^-$ . Then the buffering of the whole system is the sum of its two components, or

$$\beta = \beta_{H_2CO_3} + \beta_{Ha}$$

In the carbonate component of the buffering system the values of  $BHCO_3^-$  and  $H_2CO_3$  are the factors which must be known to evaluate buffering power. These values may be substituted in Van Slyke's equation

$$\frac{dB}{dpH} = \frac{2.3 [BHCO_3^-] \times [H_2CO_3]}{[BHCO_3^-] + [H_2CO_3]}$$

in order to show the value of  $\beta_{H_2CO_3}$ .

Since the values substituted vary with  $pCO_2$ , it is necessary to select those which are appropriate to each pH considered. Buffering values have been calculated for Curve II at pH 7.0, 6.83, and 6.65, and are shown in Table III. The carbonate buffering values calculated from the different curves are approximately equal.

The value of  $\beta_{Ha}$  cannot be calculated in the same way, because the concentrations of  $Ha$  and  $Ba$  are not known. But the concen-

$\text{CO}_2$  Dissociation Curve

tration of the anions "a" is constant, and its buffer system behaves as does the salt of a weak acid to which an acid is added. The acid added is in this case  $\text{H}_2\text{CO}_3$ , which is too weak to combine with Ba

TABLE III  
*Buffering Power of Muscle*

Curve No.	pH	$\beta_{\text{H}_2\text{CO}_3}$	$\frac{\beta_{\text{aH}}}{\text{pH } 7.0-6.65}$	$\text{CO}_2 \text{ escape}$
II	7.0	0.0031	0.020	0.021
	6.83	0.0053		0.028
	6.65	0.0076		0.036

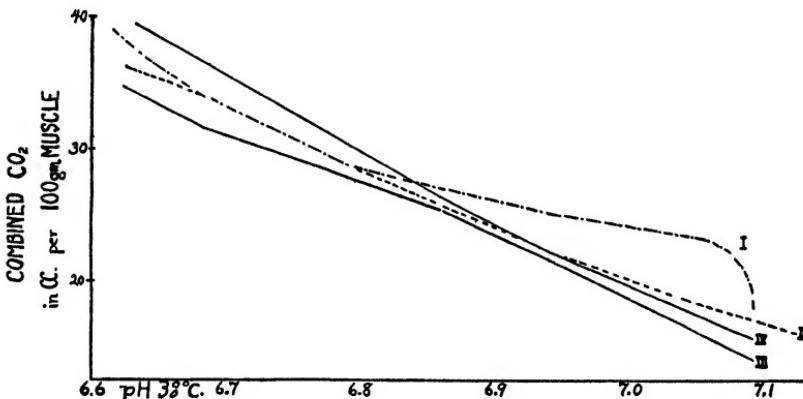


FIG. 3. From each one of the curves fitted to the experimental results (Fig. 1) the combined  $\text{CO}_2$  was estimated and plotted against the calculated pH. The change in combined  $\text{CO}_2$ ,  $(\Delta\text{BHCO}_3) = \Delta B$  for the weak acid buffers other than  $\text{H}_2\text{CO}_3$ ; consequently the slopes of these curves give  $\frac{dB}{dpH}$  or the buffer powers of the weak acids other than  $\text{H}_2\text{CO}_3$ , and enable one to see at a glance the conclusions regarding buffer power which are implied in each one of the curves which may be adopted to represent our results. Curve I seems improbably erratic. The other three point to a nearly uniform buffer power of about 0.02 in the range studied.

mol for mol. But the amount of base displaced from Ba is equal to  $\Delta\text{BHCO}_3$ . Then  $\Delta\text{BHCO}_3 = -\Delta\text{Ba} = -\Delta B$  and the buffering of the weak acids other than  $\text{H}_2\text{CO}_3$  is

$$\frac{\Delta B}{\Delta \text{pH}} = \frac{-\Delta \text{Ba}}{-\Delta \text{pH}} \quad \beta_{\text{Ha}}$$

At the average pH 6.83, the buffer values are indicated in Table III. The average buffering due to Ha as calculated from the four curves is about 0.02, which is 4 times greater than the greatest possible due to buffering by the carbonates at that pH. The range of Curves II, III, and IV shown in Fig. 3 indicates that the buffering by the other acids is probably about uniform. Since it is known that several weak acids participate in the buffering, it is unlikely that there is a sharp maximum.

A third mechanism for regulating the acid-base equilibrium must be considered. If a strong acid be added to a muscle in the case in which by means of perfect adjustment of the circulation the  $\text{CO}_2$  tension is maintained constant, the carbonic acid formed by the action of the strong acid on bicarbonate is lost from the system. The condition is no longer one of true carbonic acid buffering in which  $\text{H}_2\text{CO}_3$  replaces a stronger acid but becomes one in which base is released by loss of  $\text{CO}_2$ . The effectiveness of this mechanism as a buffer is much greater than that of true  $\text{H}_2\text{CO}_3$  buffering in muscle and it can be estimated as follows:

$$\text{pH} = \text{pK}' + \log [\text{BHCO}_3] - \log [\text{H}_2\text{CO}_3]$$

Since  $[\text{H}_2\text{CO}_3]$  is a constant because  $p\text{CO}_2$  is kept constant

$$d\text{pH} = d \log [\text{BHCO}_3] = \frac{0.4343}{[\text{BHCO}_3]} d[\text{BHCO}_3]$$

But  $d[\text{BHCO}_3] = dB$

$$\therefore \frac{dB}{d\text{pH}} = 2.3 [\text{BHCO}_3]$$

By means of this expression values have been calculated for buffer power by loss of  $\text{CO}_2$  and are recorded in Table III.

Table III shows the greatest buffer power which can be exerted by each of the three systems outlined above at a given pH, and under the conditions obtaining at the point on the  $\text{CO}_2$  dissociation curve which has that pH. In the body the buffer power of the muscle as a whole is not the sum of these three. For one reason, the buffering by  $\text{H}_2\text{CO}_3$  varies inversely as the buffering by  $\text{CO}_2$  escape. In any given situation neither can exert its full power except when the other is not operating. For it is obvious that if

H<sub>2</sub>CO<sub>3</sub> is kept constant by CO<sub>2</sub> loss there can be no true H<sub>2</sub>CO<sub>3</sub> buffering.

It is difficult to determine the condition of the bicarbonates when the buffering power is measured by titration. In the experiments of Furusawa and Kerridge (1927) the determinations were made at low temperature with some precautions against the escape of CO<sub>2</sub>. Without CO<sub>2</sub> loss our figure for  $\beta$  at the average pH 6.84 is 0.025 (sum of the third and fourth columns, Table III), compared with theirs of 0.037. Their greater buffering may have been caused by some escape of CO<sub>2</sub> or possibly a change in the thawing muscle.

Fenn (1928) estimated the buffering power of frog muscle from the CO<sub>2</sub> dissociation curve and arrived at figures about one-third as great as ours at pH 6.8 (temperature 22°).

It may be suggested that the buffering of the dog muscles used would change if phosphocreatine were decomposed by the higher CO<sub>2</sub> tension. Lipmann and Meyerhof (1930) have found that CO<sub>2</sub> does favor the splitting of phosphocreatine, and that process would be expected to release base. We have not controlled the possibility, but there is no evidence that any other buffering systems are involved than the ones which we have described. The slope of Curve II and value of its derivatives are consistent with the view of a mixed but unchanging system of weak acids and their salts.

In this paper no attempt has been made to discuss the buffering in worked muscle. Circulated muscles, in which lactic acid has increased measurably as the result of work, show considerable changes in water content, which greatly complicate the problem.

#### SUMMARY

The CO<sub>2</sub> dissociation curve of resting dog muscle may be expressed by the equation, CO<sub>2</sub> in cc. per 100 gm. =  $3.4\sqrt{pCO_2}$ .

From this curve, with assumption of the value  $\alpha$  gm. = 0.41, a combining power curve has been constructed.

With the further assumption of a value pK'<sub>1</sub> = 6.07, pH values have been calculated and used for estimating buffering power of muscle.

The buffering has been resolved to the following components (1) the carbonic acid and bicarbonates; (2) the other (non-vola-

tile) weak acids and their salts; (3) the escape of  $\text{CO}_2$  when buffering occurs at constant  $p\text{CO}_2$ .

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## THE DETERMINATION OF URIC ACID IN HUMAN URINE

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Improvements in the methods for the determination of uric acid, during the last 10 years, have been concerned primarily with the determination of uric acid in blood. The last significant contribution dealing with the determination of uric acid in urine was made by Benedict and Franke (1) in 1922, with the introduction of a direct colorimetric method. Their procedure involves the production of a color with urine for colorimetric comparison without the preliminary separation of the uric acid, as recommended in the earlier methods.

Benedict and Franke (1) compared the uric acid content of 50 samples of human urine as determined by this direct method and the older indirect method<sup>1</sup> of Benedict and Hitchcock (2), which involved a preliminary precipitation of the uric acid as a silver salt. Since the agreement in most cases was within 2 or 3 per cent, there seemed to be no objection to the use of the direct method for the analysis of human urine. Apparently many subsequent workers, regardless of the nature of the experimental work, have employed the direct method without checking the results by one of the older standard methods.

Christman and Mosier (3) have recently demonstrated that uric acid determinations by the direct method of Benedict and Franke are not accurate if the urines contain appreciable amounts of amino nitrogen (*e.g.*, glycine). The presence of amounts of glycine furnishing 0.046 to 0.373 mg. of amino nitrogen in the

<sup>1</sup> Hereafter in this paper, methods for uric acid which involve a preliminary separation of the uric acid from other urinary constituents, prior to the colorimetric determination, will be designated as "indirect" methods.

## Uric Acid Determination

uric acid standard resulted in the production of a color by the Benedict-Franke procedure which had an intensity of 10 to 40 per cent less than that of the standard color. Since the above data were reported, the experiments with glycine have been repeated. The same sample of glycine was used but different samples of sodium cyanide and arsenotungstic acid reagent were used. It was observed that with these reagents the presence of 0.373 mg. of amino nitrogen in the uric acid standard produced an error of 30 rather than 40 per cent as previously observed. Since

TABLE I  
*Errors in Analysis of Uric Acid by Benedict-Franke and Modified Methods in Presence of Amino Acids*

Source of amino N	Error by Benedict-Franke method				Error by modified method			
	$\alpha$ -Amino N present, mg.*							
	0.046	0.093	0.187	0.373	0.046	0.093	0.187	0.373
Glycine.....	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glycine.....	10	20	25	30	1	2	4	6
Alanine.....	7	10	19	24	2	2	4	8
Aspartic acid.....	7	10	14	20	0	3	4	7
Histidine.....	5	13	16	25	3	3	3	5
Lysine.....	15	20	27	33	1	2	9	15

\* Amounts of the amino acids which would furnish these quantities of  $\alpha$ -amino nitrogen were added directly to the flask containing the standard amount of uric acid, prior to the development of the color.

the use of other samples of cyanide, other factors remaining the same, resulted in errors intermediate between 30 and 40 per cent, it was thought that the variable composition of the sodium cyanide was responsible for these differences. Subsequently the effect of the presence of amino acids other than glycine upon the development of color in the uric acid standard by the method of Benedict and Franke was studied. These results are presented in tabular form (Table I). The presence of amounts of alanine, histidine, and aspartic acid calculated to provide 0.373 mg. of amino nitrogen led to errors of 20 to 25 per cent in the uric acid determinations. The addition of lysine in an amount calculated to give 0.373 mg. of  $\alpha$ -amino nitrogen resulted in an error of 33 per cent.

It has been noted in this laboratory during the past 4 years that occasionally the analysis of normal human urine by the direct Benedict and Franke procedure yielded results that were definitely lower than those obtained by methods involving a preliminary precipitation of the uric acid, such as the methods of Benedict and Hitchcock (2), Morris and Macleod (4), and Folin and Denis (5). The greatest discrepancy between the results obtained by the direct procedure on the one hand and the indirect procedure on the other hand was observed in the more concentrated urines. If the uric acid content of the volume of urine employed in the analysis by the Benedict and Franke method was such that the colorimetric reading of the unknown was between 13 and 17 mm., with the standard set at 20 mm., results by the direct method were invariably lower than those obtained by the indirect procedures. If the determination by the direct method was then repeated with a smaller volume of urine, so that the colorimetric reading was between 20 and 25 mm., the results more nearly approximated those of the indirect methods.

The results of a typical analysis of a normal urine by the Benedict-Franke method are given in Table II (Urine 1). It is to be noted that, when the analysis was made by the method of Benedict and Franke, the apparent amount of uric acid per liter of urine varied from 300 to 446 mg. It is to be further noted that, as the volume of urine used in the analysis was decreased, the apparent uric acid content of the urine was increased. The colorimetric readings, ranging from 13.3 to 25.6 mm., are not beyond the limits prescribed by Benedict and Franke for accurate results by this method. Results similar to these have been obtained with numerous urines. The analyses of two additional urines are presented in Table II (Urines 2 and 3).

Analyses of several normal human urines by the colorimetric method of Folin (6) showed the content of amino nitrogen to be low, usually less than 0.15 mg. per cc. of urine. Since it has been demonstrated (Table I) that the presence of amino nitrogen even in low concentrations caused considerable error in the analysis for uric acid by the Benedict-Franke method, a modification of the cyanide solution was made to minimize the effect of the presence of amino nitrogen upon the uric acid color reaction. This was accomplished by the inclusion of sodium carbonate in the

## Uric Acid Determination

TABLE II  
*Analysis of Normal Human Urine for Uric Acid*

Urine No.	Benedict-Franke method			Modified method			Benedict-Hitchcock method	Folin-Denis method	Morris-Macleod method
	Volume of urine*	Colorimetric reading		Uric acid	Volume of urine*	Colorimetric reading			
		cc.	mm.			mg. per l.	cc.	mm.	mg. per l.
1	1.00	13.3	300	300	1.00	13.0	461	435	424
	0.80	14.5	345	345	0.80	16.5	456		404
	0.50	19.7	406	406	0.50	26.4	454		
	0.40	23.6	422	422	0.35	37.8	451		
	0.35	25.6	446	446	1.00†	14.2	423		
	0.50†	19.7	406						
2	0.80	17.9	279	279	2.00	8.0	375	365	372
	0.70	18.9	303	303	1.60	10.0	375		
	0.50	23.5	340	340	1.00	15.5	387		
	0.45	26.0	342	342	0.70	22.2	386		
	0.40	28.0	377	377	0.50	30.5	392		
					0.40	38.7	388		
3	0.90	16.8	265	265	1.60	10.3	364	360	348
	0.80	17.9	279	279	1.00	16.4	366		
	0.60	21.4	311	311	0.80	20.2	371		
	0.50	24.5	326	326	0.60	27.0	370		
	0.40	28.4	352	352	0.50	31.7	378		
	1.00†	14.5	276	276	0.40	38.9	385		
4	0.80†	17.4	287	287	1.30†	13.4	345		
	0.60†	22.0	303	303	1.00†	17.6	341		
	0.50†	24.3	330	330	0.60†	28.7	348		
					0.40†	43.5	345		

\* In the direct procedures 10 cc. of diluted urine, equivalent to the volume of the original urine indicated in this column, were used in the analysis. In the indirect method, usually 1 cc. of diluted urine equivalent to the amount of urine indicated was used.

† In these determinations the colorimetric estimation was preceded by a precipitation of the uric acid with an ammoniacal silver magnesium solution.

sodium cyanide solution. Since the increased alkalinity of the carbonate-cyanide mixture invariably led to the formation of cloudy solutions during the development of color, urea was added to prevent turbidity in accordance with the experience of Folin (7) in his recent method for the determination of uric acid in blood. The alkaline solution finally adopted to replace the 5 per cent sodium cyanide of the Benedict-Franke method contains 5 per cent of sodium cyanide, 10 per cent of sodium carbonate, and 15 per cent of urea.

#### *Preparation of Reagents*

25 gm. of pure sodium cyanide and 50 gm. of anhydrous sodium carbonate are dissolved in 400 cc. of distilled water. If heat has been applied to hasten solution, cool, add 75 gm. of urea, and after solution is complete make to a volume of 500 cc. and filter. A slight precipitate will settle from this solution on standing but the value of the solution for the determination remains unimpaired for several months.

The intensity of the color produced by 5 cc. of this alkaline solution, 0.2 mg. of uric acid, and 1 cc. of Benedict's arsenotungstic acid reagent is less than that obtained when 5 per cent sodium cyanide furnishes the alkaline medium for the reaction. Accordingly, in order to obtain a color intensity comparable to that of the Benedict-Franke procedure, the concentration of the uric acid in the standard solution is increased from 0.2 to 0.3 mg. per 10 cc. of solution. Such a standard solution may be made by the dilution of the Folin (5) or the Benedict-Hitchcock (2) stock uric acid solution. In case the Benedict-Hitchcock stock solution is used, the equivalent of 0.5 cc. of concentrated hydrochloric acid should be present in each liter of the diluted standard. It has been our experience that the Folin stock solution of uric acid is more stable than that of Benedict and Hitchcock. The diluted standard solution made from the Folin stock solution has remained practically constant in value for 1 month.

The arsenotungstic acid reagent used by Benedict and Franke is retained in its original form.

Later in the paper the desirability of a preliminary separation of the uric acid from urine, prior to its colorimetric estimation, will be discussed. For this purpose the ammoniacal silver magnesium

solution recommended by Benedict and Hitchcock (2) is used in conjunction with the reagents just discussed.

*Direct Procedure for Determination of Uric Acid in Normal Human Urine*

The urine is so diluted that 10 cc. will contain between 0.15 and 0.60 mg. of uric acid. A dilution of 1:10 is usually satisfactory. 10 cc. portions of the diluted urine are measured into 50 cc. volumetric flasks. In a third flask are placed 10 cc. of the uric acid standard equivalent to 0.3 mg. of uric acid. 5 cc. of the cyanide-carbonate-urea solution are now added to each flask from a burette. After the contents of the flask have been thoroughly mixed, 2 cc. of the uric acid reagent are added to each flask and the contents again mixed. After 5 minutes the volume of liquid in each flask is made to 50 cc. with distilled water, the contents mixed thoroughly, and the color comparison made in the usual manner. The method here outlined will be referred to subsequently as the modified method.

In order to test the range of proportionality by this modified procedure, 10 cc. portions of uric acid solutions, varying in concentration from 0.15 to 0.60 mg. of uric acid have been analyzed. The depths of color obtained were found to be almost directly proportional to the concentration of the uric acid. If the color produced by 0.3 mg. of uric acid is set at 20 mm., readings in the colorimeter as high as 40 mm. and as low as 10 mm. are reliable to within 1 or 2 per cent. Experiments similar to those already discussed earlier in the paper were made to ascertain the effect of the presence of amino nitrogen upon the uric acid color development by the modified method. These results, presented in Table I, may be compared with those obtained by the original Benedict-Franke procedure. It is evident that regardless of the source or the amount of the amino acid, the effect upon the uric acid color reaction was in every case much less marked in the modified procedure as compared to that of Benedict and Franke. Thus in the presence of 0.093 mg. of amino nitrogen (an average amount of amino nitrogen per 1 cc. of normal urine) the error by the Benedict-Franke method was from 10 to 20 per cent while the same amount resulted in errors of 2 to 3 per cent by the modified method.

It is gratifying to note that the analysis of normal human urine

by the direct modified procedure yields consistent results, regardless of the amount of urine used in the analysis. In the analysis of Urine 1 (Table II) amounts of urine varying from 0.35 to 1.0 cc. were used. The colorimetric readings ranged from 37.8 to 13.0 mm. and the amounts of uric acid per liter of urine calculated from these readings were 451 and 461 mg. or a variation of approximately 2 per cent. These values should be compared to those obtained by the Benedict-Franke procedure for the same urine. By the latter method the variation in the apparent uric acid content of the same urine is from 300 to 446 mg. per liter depending upon the amount of urine used in the analysis. The analyses of Urines 2 and 3 (Table II) illustrate again that consistent results are obtained by the modified method regardless of the volume of urine used, while the results obtained by the Benedict-Franke procedure are subject to wide variations. Although similar results have been obtained by the analysis of many urines, occasionally a urine was analyzed by the Benedict-Franke procedure with excellent agreement regardless of the volume of urine used in the analysis, if the colorimetric readings were between 17 and 25 mm. On the other hand the analysis of urines collected during a high protein diet showed even greater variation in results than those discussed.

It is interesting to note that the values for uric acid by the Benedict-Franke method most nearly approximate those of the modified method and of the methods which involve a preliminary precipitation of the uric acid (methods of Benedict and Hitchcock, Folin and Denis, and Morris and Macleod), when the colorimetric readings by the Benedict-Franke method are between 25 and 30 mm. with the standard set at 20 mm. The proportionality of the color reaction by the Benedict-Franke method for amounts of uric acid which give readings in this range (25 to 30 mm.) has been studied and the results invariably indicated 2 to 3 per cent more uric acid than was actually present. On the other hand concentrations of uric acid which gave readings between 13 and 16 mm. yielded results that were 7 to 10 per cent too low. This lack of proportionality in the depth of color with pure uric acid solutions may explain some of the differences in the apparent uric acid content of urines when different volumes are used in analysis but it cannot explain variations as great as 30 per cent.

Although the agreement between the analyses of normal human urine by the modified direct method and the indirect methods is satisfactory for most purposes, it is believed that the determination of uric acid for research data should be made by an indirect method. This would be particularly true, for example, if the urines to be analyzed contained reducing sugars or polyphenols in more than normal amounts. Although Benedict and Franke (1) have stated that the presence of glucose in urine did not interfere with the determination of uric acid by the direct method, our experiments with their method indicate that the addition of glucose to normal urine to give concentrations of 0.25, 0.5, and 1.0 per cent increased the apparent amount of uric acid by 5, 12, and 19 per cent respectively. Somewhat greater errors by the modified method due to the presence of the same concentrations of glucose were observed. It would seem therefore that a preliminary precipitation of the uric acid should precede the colorimetric determinations for urines which contain more than a normal amount of reducing sugars.

While Benedict (8) reported on the effect of polyphenols in connection with the technique for blood analysis, no data were given for the urine method. Since polyphenols are known to occur in normal urines it was thought advisable to secure further data in regard to the influence of resorcinol on the color production for concentrations of uric acid comparable to those found in urine. Accordingly amounts of resorcinol varying from 0.1 to 1.0 mg. were added to the uric acid standards of the Benedict-Franke and the modified method. The colors were developed in the usual manner and matched against a simultaneously prepared standard containing no resorcinol. By the Benedict-Franke method the addition of 0.1, 0.2, 0.5, and 1.0 mg. of resorcinol to the 0.2 mg. of uric acid standard gave results which were too low by 15, 22, 30, and 50 per cent respectively. The presence of the same amounts of resorcinol in the standard yielded values for uric acid by the modified procedure which were 7, 10, 16, and 17 per cent too low. It is to be noted that the errors due to the presence of resorcinol are less by the modified method than by the Benedict-Franke method. Apparently the concentration of polyphenols in normal human urine is not great since the uric acid analyses by the direct modified procedure agree well with those obtained by the indirect methods (Table II).

The indirect procedure which has proved very satisfactory is as follows: 1 cc. of urine (2 cc. if the urine is dilute) is measured into a 15 cc. graduated centrifuge tube and 1 cc. of the ammoniacal silver magnesium solution of Benedict and Hitchcock (2) is added. This is stirred thoroughly with a small stirring rod and the precipitate is separated at once by rapid centrifugation. The supernatant liquid is carefully decanted and the lip of the tube wiped to remove the last trace of liquid. 0.5 cc. of the cyanide-carbonate-urea solution is added from a burette to the precipitate and the mixture is stirred thoroughly with a small stirring rod. 4.5 cc. more of the alkaline solution are then added and the mixture is stirred until the precipitate is completely dissolved. This solution is then transferred with sufficient washing to a 50 cc. volumetric flask to give a final volume of 15 cc. The development of the color and comparison with the standard color are completed as described for the direct modified method.

The analysis of a large number of normal human urines for uric acid by this and the direct modified procedure indicates that the latter method gives results which are 5 to 8 per cent higher than the indirect method. In the case of Urine 3, various volumes of urine were used in the indirect analysis by the modified method. Practically identical results were obtained although the colorimetric readings ranged from 13.4 to 43.5 mm. The analysis of the same urine by the Benedict-Franke method, preceded by a preliminary precipitation of the uric acid, gave values varying from 276 to 330 mg. of uric acid per liter, depending upon the volume of urine employed in the analysis. In view of this variation in results a further study will be made to determine whether the substances in urine, which interfere with the Benedict-Franke direct procedure, are partially precipitated with the uric acid.

#### DISCUSSION

Myers and Wardell (9) in their extensive use of the Benedict-Franke method have apparently experienced none of the difficulties which are reported in this paper. In fact, these authors, on the basis of their uric acid isolation experiments, stated that the accuracy of the Benedict-Franke method had been substantiated. It is interesting to note in the report of Myers and Wardell that many of the normal human urines indicated a definitely higher

content of uric acid by the indirect Benedict-Hitchcock method than by the direct method of Benedict and Franke. Similar observations in this laboratory led to the present study of the uric acid methods.

It is possible that the sodium cyanide used by Myers and Wardell in the Benedict-Franke method contained more sodium carbonate than the cyanide used in this laboratory. If this were the case, the Benedict-Franke method would give more accurate results than reported in this paper. Different lots of pure sodium cyanide (Mallinckrodt) were used throughout this work. The experiments recorded in this paper were practically completed before our attention was called to the fact that various brands of sodium cyanide might contain varying amounts of sodium carbonate. Consequently no attempt was made to determine the sodium carbonate content of the various sodium cyanide preparations.

Benedict (10) has criticized the use of acid silver solutions as precipitants for uric acid because of the lack of specificity as compared to ammoniacal silver solutions. Apart from the question of specificity, there is an objection to the use of the acid silver lactate precipitation reagent, as used in the Folin-Wu (11) method for the determination of uric acid in blood and the Folin-Denis method for the determination of uric acid in urine. Rogers (12) reported a number of years ago that serious errors in the determination of uric acid in blood by the Folin-Wu method may result, if the silver precipitate containing the uric acid is exposed for a short time to the ordinary light of a well lighted laboratory. Our experience with the Folin-Denis method for urinary uric acid confirms the observation of Rogers with regard to the rapid loss of uric acid from the silver precipitate on exposure to bright light. Much of the work in regard to this point was done during the late spring and summer months in a well lighted laboratory with a western exposure. It was found that on clear, sunny days delay in redissolving the silver precipitate containing the uric acid invariably led to low results, even though the precipitates were not exposed to direct sunlight. On dark, cloudy days the uric acid in the precipitates remained unchanged for several hours, but if the precipitates were allowed to stand for periods of 12 to 18 hours in comparatively dark cupboards, errors of 30 to 40 per cent would be noted.

The change in color of the precipitate from a light gray to a deep brown or black during this period should serve as a warning to a trained analyst. In view of these facts, the desirability of completing the determination as quickly as possible after the precipitation of the uric acid, without undue exposure to light, cannot be overemphasized.

Simultaneously conducted experiments indicate that the uric acid precipitated by ammoniacal silver solutions as used in the proposed indirect method is not affected by an exposure of several hours on a clear day, provided the precipitate is not exposed to direct sunlight.

#### SUMMARY

Attention is directed to the wide variation in results obtained in the analysis of normal human urine for uric acid by the Benedict-Franke method, depending upon the volume of urine used in the analysis. A modification of the direct procedure which yields more consistent results is proposed. For a more accurate analysis of uric acid in human urine, a modified procedure involving a preliminary precipitation of the uric acid prior to its colorimetric estimation is recommended.

*Addendum*—Since this paper was accepted for publication, the last number of the *Biochemical Journal* has been received. Salt (13) has modified the method of Folin for the determination of uric acid in urine to prevent turbidity during the development of the color. This was accomplished by the use of a cyanide-urea solution and a 10 per cent rather than a 20 per cent sodium carbonate solution as used in the original procedure. During the past year many determinations of uric acid in urine have been made in this laboratory by the Folin procedure, without the troublesome turbidity during the color development, by the inclusion of urea in the 20 per cent sodium carbonate to the extent of 15 per cent. The results by this modification were identical with those by the original Folin procedure.

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## SOME PHYSICOCHEMICAL PROPERTIES OF SPECIFIC POLYSACCHARIDES\*

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In recent years the study of immunologically specific polysaccharides has not only contributed to the understanding of bacterial specificity (1) but has also proved of purely chemical interest because of the unusual structure of certain members of the group. Thus the specific polysaccharide of Type III pneumococcus has been shown to be a polyaldobionic acid built up of -glycurono-glucose units (2, 3). It is, moreover, a typical colloid in the sense that it does not diffuse through collodion or parchment into water, and is, by virtue of its structure, so strong an acid that its aqueous solutions turn Congo red paper blue. Hence it seemed of interest to study some of the physicochemical properties of solutions of its salts, the more so as little is known of the behavior to be expected of an organic, colloidal, strong electrolyte devoid of basic groups. Accordingly, the sodium salt of the Type III pneumococcus specific polysaccharide was subjected to a study of its viscosity, conductance, and its behavior on diffusion. Measurements were also made on solutions of other specific polysaccharides. The diffusion data are reserved for a forthcoming paper dealing with the molecular weight of specific polysaccharides, in which connection the data recorded in the present communication will again be taken up.

Physicochemical studies have previously been made of gum arabic, a carbohydrate of a character somewhat analogous to that of the Type III pneumococcus polysaccharide. This substance, however, is the salt of a much weaker acid than the Type III

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pneumococcus carbohydrate, since the equivalent weight of arabic acid is about 1200, while the Type III substance (hereinafter referred to as S III) possesses one carboxyl group for every 340 of molecular weight. Thomas and Murray (4) have given a titration curve of arabic acid and also studied its osmotic pressure and viscosity at various hydrogen ion concentrations, and concluded that its behavior might be satisfactorily explained on the basis of the

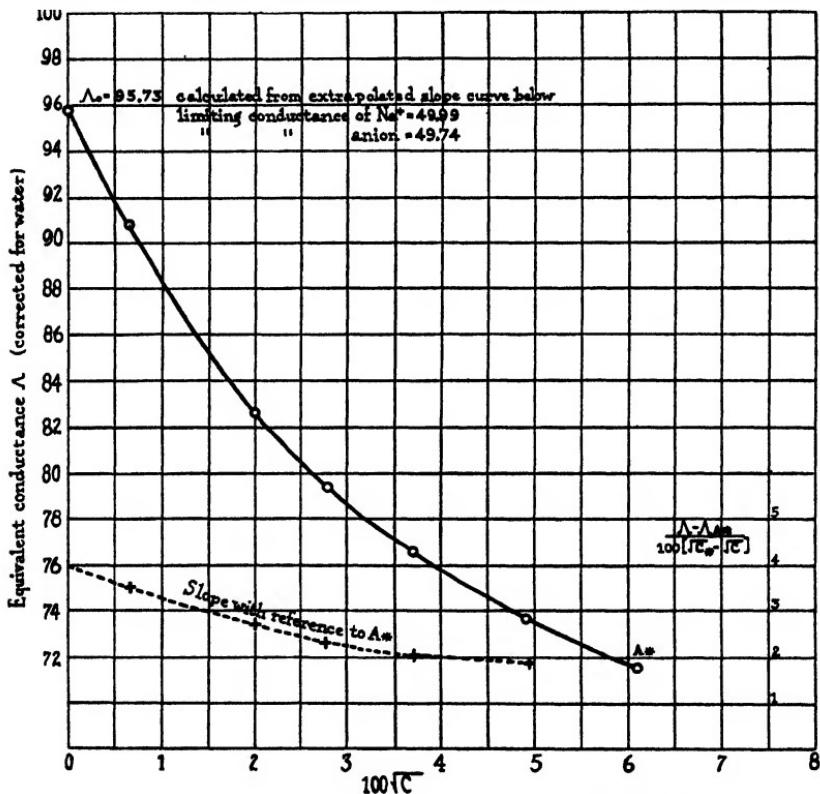


FIG. 1. Conductance of sodium salt of pneumococcus Type III specific polysaccharide.

Donnan equilibrium. The application of this conclusion to the viscosity data was rejected by Taft and Malm (5) on the basis of an extended study which included conductivity measurements. These resulted in the conclusion that the current was carried mainly by the inorganic ions present.

TABLE I  
Conductivity of Na Salt of S III at  $25^\circ \pm 0.003^\circ$

Total weight of stock solution added	Equivalents $\times 10^{-4}$	Volume (at 25°) ml.	$C \times 10^4$ equivalents	$100 \sqrt{C}$	Resistance reading in bridge ( $R$ ) ohms	$\frac{10^4}{R}$	$\frac{10^4}{R'}$	Uncorrected specific conductance ( $L'$ ) $\times 10^4$	Specific conductivity corrected for water (L) $\times 10^4$	Equivalent conductance (A)
gm.					ohms			mhos	mhos	mhos
0	0	929.7	0	9917.0	0.00837	0.01018	0.005996	0.037940	90.863	
1.0631	3.8862	930.7	0.041755	0.647	9321.5	1.07279	0.07460	0.043936	0.34295	82.571
10.6849	39.059	940.4	0.41534	2.04	6286.6	1.59069	0.59250	0.34895	0.61820	79.385
20.2357	73.972	949.9	0.77873	2.79	4859.0	2.05804	1.0599	0.62420	1.0431	76.575
35.9751	131.507	965.4	1.3622	3.69	3597.8	2.77948	1.7813	1.0491	1.7994	73.697
66.4993	243.088	995.6	2.4416	4.94	2460.8	4.06372	3.0655	1.8054	2.6690	71.609
105.4481	385.466	1034.2	3.7272	6.11	1805.0	5.54017	4.5420	2.6750		

Concentration of stock solution,  $3.6555 \times 10^{-4}$  equivalents  
gm. solution in air.

Equivalent weight, 340.

The cell was shunted by a resistance coil (S) of 10018.1 ohms in the measurements.  
 $R'$ , bridge reading.

$R'$ , actual resistance of cell.  $K$ , cell constant = 0.58895.

$$\frac{1}{R'} = \frac{1}{R} - \frac{1}{S}; L' = \frac{K}{R'}; L = L' - L_{H_2O}; \Lambda = \frac{1000 L}{C}.$$

## EXPERIMENTAL

*Conductivity Determinations*—The conductivity data were obtained with the arrangement of apparatus described by Shedlovsky (6). An S III solution was added to a new type of cell (7) from a

TABLE II  
Viscosities of S III Measured with Bingham Viscometer, No. 123,  
 $K = 1.99 \times 10^{-5}$

Temperature °C.	Concentration per cent	Solvent	$\mu$ (corrected)	Time sec.	$\eta$ cen'i- poises	$\eta_{\text{solvent}}$ cen'i- poises	$\eta_r$	$\eta_{sp}$	$\frac{\eta_{sp}}{C}$
20	2.93	H <sub>2</sub> O	188.90	6859	25.78	1.0050	25.65	24.65	8.41
	0.293	"	117.39	1390.6	3.248	1.0050	3.232	2.232	7.62
	0.293	"	216.4	752.4	3.234	1.0050	3.218	2.218	7.57
	0.0293	"	214.05	352.7	1.503	1.0050	1.495	0.495	16.89
	0.0293	"	99.18	799.7	1.539	1.0050	1.531	0.531	18.12
	0.0293	"	280.3	266.1	1.484	1.0050	1.477	0.477	16.28
	0.0293	"	99.12	617.9	1.219	0.8007	1.522	0.522	17.82
30	0.0293	"	208.99	286.4	1.191	0.8007	1.488	0.488	16.66
	0.0293	"	280.32	211.3	1.179	0.8007	1.472	0.472	16.11
	0.0293	0.17 M NaCl	280.04	192.8	1.074	1.0090	1.064	0.064	2.18
20	0.0293	0.17 " "	193.23	278.7	1.072	1.0090	1.062	0.062	2.12
	0.0293	0.17 " "	110.87	487.0	1.075	1.0090	1.065	0.065	2.22
	0.0293	0.17 " "	111.08	388.3	0.858	0.809	1.061	0.061	2.08
30	0.0293	0.17 " "	212.37	202.8	0.857	0.809	1.059	0.059	2.01
	0.0586	0.17 " "	233.86	243.7	1.134	1.009	1.124	0.124	2.12
	0.0586	0.17 " "	132.31	431.4	1.134	1.009	1.124	0.124	2.12
20	0.0293	0.85 " "	132.25	426.9	1.124	1.078	1.042	0.042	1.43
	0.0293	0.85 " "	166.79	338.4	1.123	1.078	1.042	0.042	1.43
	0.0293	0.85 " "	219.27	257.8	1.124	1.078	1.043	0.043	1.47

weight burette, so that increasing concentrations were measured without emptying the cell. The equivalent weight was taken as 340 and measurements were made in the range 0.039 to 3.85 milliequivalents. The extrapolation to limiting conductance was made with the aid of a derived slope curve by use of the formula given in Fig. 1. The data are recorded in Table I and Fig. 1.

That the results were not influenced by hydrolysis is indicated by

the fact that no shift in pH could be observed with either methyl red or phenol red as indicator on diluting a 30 mm solution 100 times with freshly boiled distilled water.

TABLE III  
*Viscosities of S III Solutions. Ostwald Viscometer at 20°*

Substance	Solvent	Concen- tration	Time	Time, sol- vent	$\eta_r$	$\eta_{sp}$	$\frac{\eta_{sp}}{C}$	$\frac{\eta_{sp}}{\sqrt{C}}$
		per cent	sec.	sec.				
$H_2O$ S III	$H_2O$		92.6					
		0.293	303*	92.6	3.27	2.27	7.75	4.19
		"	0.0586	174.8	92.6	1.89	0.89	15.20
		"	0.0293	147.9	92.6	1.60	0.60	20.48
		"	0.0195	137.1	92.6	1.48	0.48	24.62
		"	0.01465	132.5	92.6	1.43	0.43	29.35
KCl	$H_2O$	1.21 M	86.6				$\frac{\eta_{sp}}{C}$	corrected for vol- ume of KCl
		1.21 M KCl	0.2930	129.9	86.6	1.50	0.50	1.71
		1.21 " "	0.0293	90.5	86.6	1.05	0.05	1.71
		KCl	$H_2O$	3.35 M	82.4			
		S III	3.35 M KCl	0.293	99.4	82.4	1.21	0.21
$Na_2HPO_4$	$H_2O$	0.05 M	95.5				0.72	0.64
S III	$H_2O$	0.05 M $Na_2HPO_4$	1.00	406.7	95.5	4.26	3.26	3.26
HCl	$H_2O$	0.05 "	0.10	117.0	95.5	1.23	0.23	2.30
S III	$H_2O$	0.27 M HCl	0.0293	97.7	94.1	1.04	0.04	
HCl	$H_2O$	2.60 M	101.5					
S III	$H_2O$	2.60 M HCl	0.0293	106.1	101.5	1.05	0.05	
NaCl	$H_2O$	0.17 M	93.7					
S III	$H_2O$	0.17 M NaCl	0.0293	99.6	93.7	1.06	0.06	

\* Corrected for relative density of solution,  $d_{40}^{20} = 1.003$ ;  $T_{obs.} \times d$ .

The conductivity determinations were made at The Rockefeller Institute for Medical Research by Dr. T. Shedlovsky, and it is a pleasure for the writers to express their indebtedness to him for his freely given time and invaluable aid in the interpretation of the

data. Thanks are also due Dr. Duncan A. MacInnes of the Rockefeller Institute for his interest and assistance.

TABLE IV  
*Viscosities of S I and S II in Ostwald Viscometer at 20°*

Substance	Solvent	Concen-tration	Time	Time, solvent	$\eta_r$	$\eta_{sp}$	$\frac{\eta_{sp}}{C}$	$\frac{\eta_{sp}}{\sqrt{C}}$
$H_2O$ S I	$H_2O$		92.9					
		0.3273	187.9*	92.9	2.05	1.05	3.21	1.84
		"	0.06546	122.3	92.9	1.32	0.32	4.89
		"	0.03273	108.7	92.9	1.17	0.17	5.20
		"	0.02181	105.6	92.9	1.14	0.14	6.42
		"	0.01637	103.2	92.9	1.11	0.11	6.72
		"	0.0131	100.5	92.9	1.08	0.08	6.11
KCl	$H_2O$	1.34 M	86.0				$\frac{\eta_{sp}}{C}$	corrected for volume of KCl
S I	1.34 M KCl	0.3273	116.5	86.0	1.35	0.35	1.07	1.03
	3.35 " "	0.3273	109.6	82.4†	1.33	0.33	1.01	0.90
	0.17 " NaCl	0.3273	132.8	93.7†	1.42	0.42	1.28	
	0.27 " HCl	0.3273	141.4	94.1†	1.50	0.50	1.53	
	2.60 " "	0.3273	153.0	101.5†	1.51	0.51	1.56	
	0.008 M HCl	0.3273	164.5	92.6†	1.78	0.78	2.38	
	0.009 " "	0.2975	146.2	92.6†	1.58	0.58	1.95	
$H_2O$ S II	$H_2O$	1.106	113.7‡	93.1	1.22	0.22	0.20	
NaCl	"	0.221	97.2	93.1	1.04	0.04	0.18	
S II	0.85 M NaCl	0.553	103.2	96.5	1.07	0.07	0.13	

\* Corrected for relative density of solution,  $d_{20}^{20} = 1.002$ .

† From Table III. Time for  $H_2O$  is used for 0.008 and 0.009 M HCl.

‡ Corrected for relative density of solution,  $d_{20}^{20} = 1.007$ .

**Viscosity Measurements**—The viscosity data given in Table II were determined as described by Bingham (8) with the Bingham viscometer at Lafayette College, and the writers wish to thank Professor E. C. Bingham for extending to them the hospitality of his laboratory and the benefit of his personal interest. All pres-

sures were corrected for temperature, hydrostatic head, and flow. The viscosities were expressed in absolute units and the relative and specific viscosities calculated.

The remaining viscosities were determined with an Ostwald viscometer at 20°, the value obtained with an S III solution within the range of the Hagen-Poiseuille law checking closely with that found with the aid of the Bingham instrument. 5.0 cc. of solution were used in every case. Potassium chloride was used instead of sodium chloride at the higher salt concentrations since its effect on the viscosity of water is almost negligible.

TABLE V  
*Viscosities of Specific Polysaccharides in Ostwald Viscometer at 20°*

Substance	Concen- tration	Time	<i>d</i>	<i>T</i> × <i>d</i>	Time, solvent	$\eta_r$	$\eta_{sp}$	$\frac{\eta_{sp}}{C}$
Specific gum arabic (S.G.A.)								
H <sub>2</sub> O	per cent	sec.			sec.			
S.G.A.	4.85	143.2	1.022	146.4	93.1	1.57	0.57	0.12
"	0.485	99.0	1.002	99.2	93.1	1.07	0.07	0.14
"	0.0485	94.0			93.1	1.01	0.01	0.21
Bovine tubercle bacillus polysaccharide (B.B.G. 526 I A*)								
H <sub>2</sub> O		92.6						
B.B.G.	1.218	102.1	1.003	102.4	92.6	1.11	0.11	0.09
"	0.1218	93.9			92.6	1.01	0.01	0.08

\* To be described in a later publication.

The results are given in Tables III to V and in part in Fig. 2.

*Titration of Type I Pneumococcus Specific Polysaccharide*—It will be recalled that S I is both a weak base and strong acid (9). Since the isoelectric substance dissolves only slowly in alkali the titration was carried out as follows: A weighed sample of anhydrous S I was wet with water and dissolved with a measured amount of N/14 hydrochloric acid. The solution was then titrated back through the isoelectric point with N/14 sodium hydroxide. The finely divided precipitate dissolved readily at a reaction which was still acid to litmus, and the titration was continued to alkalinity

to phenolphthalein or thymolphthalein, very little additional alkali being required between the turning points of the two indicators.

0.3153 gm. of S I 58, dissolved in 10.03 cc. of N/14 HCl, required 25.23 cc. of N/14 NaOH (0.15 cc. being deducted as indicator blank) to blue color to thymolphthalein, or 15.20 cc. in excess, equivalent to 1.086 cc. of N NaOH. Acid equivalent, 290.

0.4048 gm. of S I 59 B, dissolved in 10.03 cc. of N/14 HCl, required 27.28 cc. of N/14 NaOH to bright pink to phenolphthalein,

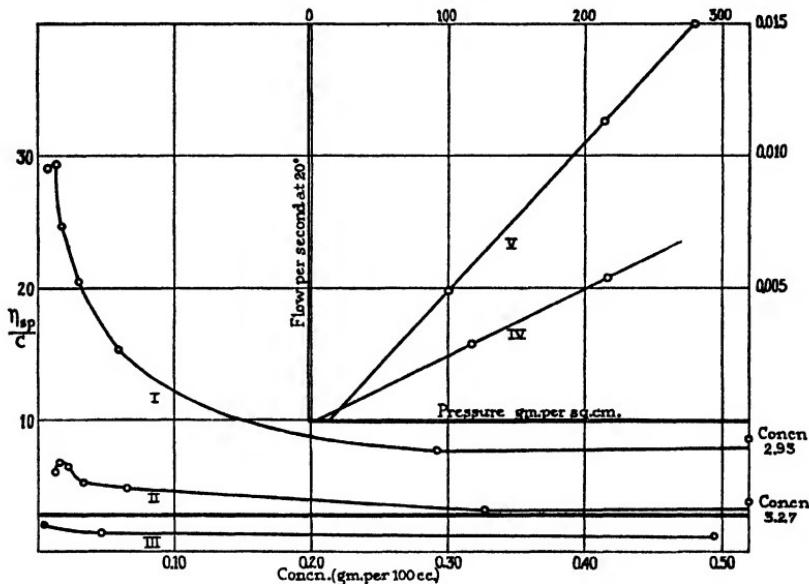


FIG. 2. Viscosity relations of sodium salts of specific polysaccharides. Curve I, S III; Curve II, S I; Curve III, S. G. A. (ordinates  $\times 0.1$ ; abscissæ  $\times 10$ ); Curves IV and V, S III 0.293 and 0.0293 per cent, respectively.

or 17.25 cc. in excess, equivalent to 1.232 cc. of N NaOH. Acid equivalent, 328. Mean acid equivalent, 309.

#### DISCUSSION

The high values of the equivalent conductance shown in Table I and Fig. 1 indicate that the sodium salt of S III is a strong electrolyte, since  $\Lambda$ , which is 95.7 at infinite dilution, drops to only 71.6 at a concentration of 3.85 milli-equivalents.

The conductivity of S III, however, does not follow the linear relationship between  $\Lambda$  and the square root of the concentration,  $\Lambda = \Lambda_\infty - a \sqrt{C}$ , derived empirically by Kohlrausch and given theoretical significance by Debye and Hückel and by Onsager (10). In a highly ionized substance, the slope of the conductance curve,  $a$ , depends largely on the valence type, increasing with the valence. In the case of an electrolyte which is incompletely dissociated, such as thallium chloride (*cf.* (10)), dilution decreases the slope, owing to the increase in the number of conductors, and the resulting curve is concave toward the origin. In the S III salt, however, this effect is more than counterbalanced, and the curve becomes convex toward the origin. This may be explained by assuming, with increasing dilution, the formation of ions of higher and higher valence type as additional COONa groups dissociate. The possibility of this taking place is shown by the structural formula for the S III salt given below in another connection. Thus, the slope,  $a$ , of the conductivity curve would tend to increase with dilution, rather than decrease, owing to the preponderating influence of the increasing valence. That even a simpler substance may show a similar deviation from a straight line relationship is readily demonstrated by plotting the equivalent conductance curves calculated from Noyes and Lombard's measurements for the tetra and penta sodium salts of benzene pentacarboxylic acid (11).

Since the limiting conductance of the sodium ion is 50.0, that of the Type III polycarboxylate ion (equivalent weight 340) would be  $95.7 - 50.0$ , or 45.7, indicating that it is a ready carrier of electricity. Its behavior is thus very different from that ascribed to a polysaccharide anion such as that of gum arabic (equivalent weight 1200) by Taft and Malm (5), who consider the inorganic ions responsible for the entire current carried. The value of the limiting conductance is, indeed, very close to recent values ascribed to the caseinate (equivalent weight 2000) (12) and globulinate ions (equivalent weight 3000) (13). The S III polycarboxylate ion, however, is free from basic groups and has a carboxyl group for every 340 of molecular weight. Since all of the available evidence indicates that there are at least eight to ten such groups in the molecule, the cumulative effect of the negative charges on the S III ion would be very large. That a total charge of this magnitude would result in large interionic or Coulomb forces can readily be appreciated.

## Specific Polysaccharides

Tables II and III show that although the specific viscosity,  $\eta_{sp} = \eta_r - 1$ , is independent of a  $10^\circ$  increase in temperature, indicating that there is no association or dissociation on changing the

TABLE VI  
*Specific Volumes of Dissolved Specific Polysaccharides According to Kunitz's Formula*

Substance	Solvent	Concen- tration per cent	$\eta_r$	$\varphi$	Specific volume cc. per gm. solute	$\frac{\eta_{sp}}{C}$
S III	H <sub>2</sub> O	0.293	3.27	23.5	80	
	"	0.0586	1.89	13.4	230	
	"	0.0293	1.60	10.0	340	
	"	0.0195	1.48	8.4	430	
	"	0.01465	1.43	7.7	530	
	"	0.01172	1.34	6.3	540	
	1.21 M KCl	0.2930	1.50	8.7	30	
	1.21 " "	0.0293	1.05	1.1	38	
	3.35 " "	0.2930	1.21	4.2	14	
	3.35 " "	0.0293	1.02	0.44	15	
	0.05 " Na <sub>2</sub> HPO <sub>4</sub>	1.00	4.26	28.3	28	
	0.05 " "	0.10	1.23	4.6	46	
S I	H <sub>2</sub> O	0.3273	2.05	14.9	46	
	"	0.0327	1.17	3.5	110	
	"	0.0218	1.14	3.0	140	
	"	0.0131	1.08	1.8	140	
	1.34 M KCl	0.3273	1.35	6.5	20	
S II	3.35 " "	0.3273	1.33	6.2	19	
	H <sub>2</sub> O	1.106	1.22	4.4	4	
	"	0.221	1.04	0.87	4	
S.G.A.	0.85 M NaCl	0.553	1.07	1.6	3	
	H <sub>2</sub> O	4.85	1.57	9.6	2	
	"	0.485	1.07	1.6	3	
Na benzene pen- tacarboxylate (Noyes and Lombard)	"	2.04	1.09	2.0	1.0	0.044
	"	0.816	1.04	0.87	1.1	0.049
	"	0.245	1.015	0.33	1.3	0.061

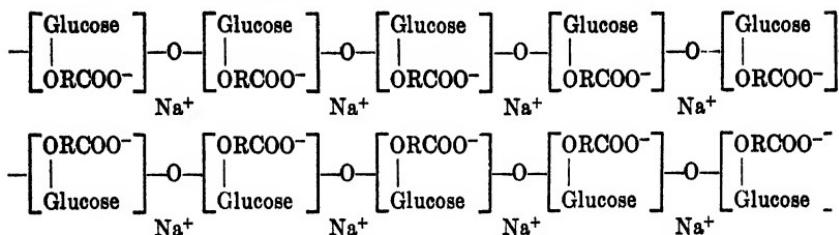
temperature in this range, aqueous solutions of S III obey neither the Hagen-Poiseuille law, nor the Einstein relation,  $\eta = \eta_0 \left(1 + K \frac{N\varphi}{V}\right)$ , from which  $\frac{\eta_{sp}}{C}$  should equal a constant. It is clear

(Fig. 2) that the latter function increases to a maximum with increasing dilution, just as the equivalent conductance increases with dilution. It is, therefore, possible to ascribe the increasing deviation from the Einstein viscosity relation to the same cause; namely, the ionization of additional COONa groups and the resultant increase in the charge on the polycarboxylate ion. In this instance the relation  $\frac{\eta_{sp}}{\sqrt{C}}$  is found to hold over a limited range of concentration, but this is not so evident in the other specific polysaccharides studied. Moreover, the viscosity determinations made by Noyes and Lombard (11) on solutions of sodium benzene pentacarboxylate show a 40 per cent increase in  $\frac{\eta_{sp}}{C}$  with decreasing concentration from 2 per cent (50 mm) to 0.25 per cent (6 mm) (Table VI). Although this is small compared with the effect noted in the case of S III, the negative charge due to the piling up of carboxyl groups on even a substance of low molecular weight appears to result in a viscosity effect in the same direction.

It is also possible to ascribe the observed viscosity effects to hydration of the specific substance. If this be true, the hydration must be of a most unusual magnitude. Otherwise, at the low concentrations of S III at which  $\frac{\eta_{sp}}{C}$  is still increasing, the concentration of free water in the system would be practically constant. If hydration be the cause of the observed anomalies, it should be possible to calculate its extent by means of Kunitz's modification (14),  $\eta = \frac{1 + 0.5\varphi}{(1 + \varphi)^4}$ , of Einstein's formula, and this does indeed lead to enormous values for the specific volume in dilute aqueous solution (Table VI), 1 gm. of S III being capable, according to the formula, of combining with as much as 540 gm. of water.

It is also shown in Fig. 2 that if the rate of flow per second of dilute solutions of S III in water is plotted against the pressure, a straight line is obtained for each concentration. These lines do not pass through the origin, but intersect the axis at a positive value for  $P$  which increases with dilution. Thus a certain pressure must be exceeded before flow starts, and the solution behaves as a plastic solid. This might be accounted for by assuming a definite orientation of the highly charged polycarboxylate ion at high dilutions

at which dissociation is approaching completion. This would also entail an orientation of the accompanying sodium ions, and a definite structure would result, somewhat as follows:



in which  $\text{RCOO}^-$  represents the glycuronic acid residue in glucosidic union with the glucose residues (*cf.* (3)). Such an arrangement should also result in a maximum value of  $\frac{\eta_{sp}}{C}$  and is consistent with the explanation given for the positive deviation from the square root conductivity relation at high dilutions.

In the case of the specific polysaccharide of Type I pneumococcus the viscosity anomalies are smaller than in the case of S III, and this might be ascribed either to the smaller magnitude of the Coulomb forces engendered by the S I anion, or to the lower extent of the hydration. The acid equivalent of S I is somewhat lower than that of S III, but since there is one basic group for approximately every three carboxyl groups, the total negative charge on the S I ion would be less than that of S III, assuming the molecular weights to be approximately the same. Also, calculation of the hydration by Kunitz's formula yields lower values than in the case of S III, so that either explanation might be valid.

In the case of S II and the specific gum arabic, with their acid equivalents of approximately 1000, both viscosity and hydration are low. Since there is an apparent parallel between the total acidity of the specific polysaccharides considered and their hydration as calculated by the Kunitz formula, it is possible that the unusual hydration, or at least the unusual viscosity, of the S III and S I is conditioned by the unusual magnitude of the total negative charge on these ions.

Association of abnormally high viscosities with an acid polysaccharide has been shown qualitatively by Raistrick and Rintoul (15) in the case of luteic acid, a polysaccharide produced from

glucose by *Penicillium luteum*, Zukal. The structural unit is composed of 2 molecules of glucose and 1 of malonic acid, one of the carboxyl groups being free. Moreover, Staudinger and Kohlschütter (16) have recently continued earlier work by Staudinger and Urech (17), giving preliminary viscosity data on polyacrylic acids. They fix the relative viscosity of a 0.1 equivalent solution of a sodium salt at the enormous value of

114 and the relation  $\frac{\eta_{sp}}{C}$  at about 1140, values far higher than obtained in the case of S III. The polyacrylic salts differ from those of S III, however, in hydrolyzing readily and in the smaller equivalent conductivity of their aqueous solutions. Staudinger originally considered the high viscosity of polyacrylic acid salts to be due to hydration caused by the high ionic charge, but now ascribes it to "swarms" caused by increasing ionization. If, as seems agreed, these high viscosities are conditioned by the piling up of large Coulomb forces, regardless of the mechanism by which this is brought about, a substance such as acrylic acid, with an acid equivalent of only 86 would be expected to develop higher viscosities as the chain of carbon atoms and carboxyl groups is lengthened by polymerization, than would the aldobionic acid structural unit of S III, with its acid equivalent of 340.

Tables II and III show clearly the effect of electrolytes in diminishing the viscosity (and in Table VI the hydration) of the solutions of S III and in bringing them into agreement with the Hagen-Poiseuille law and the Einstein equation.

It is shown in Table IV that  $\frac{\eta_{sp}}{C}$  increases to a maximum with increasing dilution in the case of the sodium salt of S I as in the case of S III, but that the increase is much smaller, and that  $\frac{\eta_{sp}}{\sqrt{C}}$  is constant over a very limited range. Although titration shows that the acid equivalent of S I is lower than that of S III, the greater number of carboxyl groups in the molecule and their expected effect on the total charge and viscosity appear to be more than negated by the simultaneous presence of basic groups. However, the internal compensation of Coulomb forces is insufficient to prevent the building up of a negative charge large enough to produce viscosity effects qualitatively similar to those encoun-

tered in the case of S III (see also Fig. 2); and in S I, also, these forces are only incompletely reduced by isotonic concentrations of salts. The smaller viscosity effects might also be attributed to a lower molecular weight for S I than for S III, but no evidence is available on this point. Although the total viscosity effects of the interionic forces due to the S I anion do not reach the proportions shown by the S III ion, when these forces are depressed by large concentrations of salts or acids, the specific viscosity of S I is very close to that of S III. This is perhaps a reflection of similarities in the arrangement of the sugar and sugar acid units of which the two substances are composed.

The low acid equivalent of S I may be taken as evidence that some other explanation must be sought for the widely differing ratios between precipitin index and mouse protection in the case of Type I and Type III antisera than the suggestion of Sobotka and Friedlander (18) that the ratios are connected with differences in the acid equivalents of the two polysaccharides.

Table V shows that in the non-basic specific gum arabic (19) (S.G.A., acid equivalent 835) the accumulation of negative charges is still great enough to give rise to an increase in  $\frac{\eta_{sp}}{C}$  on dilution, while this effect is lost in the case of the specific polysaccharide of Type II pneumococcus (9) (S II, acid equivalent 1250). The specific viscosities of these polysaccharides are also lower than those found at similar concentrations of S I and S III, but are higher than that of a non-acidic (and non-basic) bovine tubercle bacillus polysaccharide (B.B.G., Table V).

In the series of specific polysaccharides studied, therefore, there appears to be a relation between the magnitude of the viscosity phenomena shown by the substance and the frequency of occurrence of carboxyl groups in the polysaccharide chain. That the large viscosity effects exhibited by salts of S III are due to the consequent piling up of negative charges in the salts of this strong multivalent acid is believed to be indicated by the data presented.

#### SUMMARY

1. The specific polysaccharide of Type III pneumococcus is shown to yield a highly ionized sodium salt characterized by a mobile negative ion of very high valence.

2. Viscosities have been determined for a number of specific polysaccharides under varying conditions of salt concentration, and the findings correlated with the magnitude of the charge on the anion.

3. So strong are the interionic, or Coulomb, forces engendered by the S III polycarboxylate ion that the ratio  $\frac{\eta_{sp}}{C}$  increases many times with dilution. At increasing dilutions S III solutions show in increasing measure the yield point phenomenon characteristic of plastic solids, an effect possibly due to the orientation of ions brought about by these forces.

4. The specific polysaccharide of Type I pneumococcus shows viscosity abnormalities of the same character, but of smaller magnitude. Although its acid equivalent is even lower than that of S III, internal compensation of negative charges by the basic groups present is believed to be the cause of the smaller effect.

5. The data on the specific gum arabic and Type II pneumococcus polysaccharide show that the viscosity effects decrease with the relative number of carboxyl groups, or negative charges, in the molecule.

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## PROPERTIES OF DOG BLOOD

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(Received for publication, December 5, 1931)

Henderson and associates (1, 2) have defined certain physicochemical properties of oxygenated human blood. These studies naturally lead one to adventures in the field of comparative physiology. In consequence, a similar study has been made of dog blood. Since the dog is used so commonly in the laboratory, it should be useful to know how closely its blood resembles that of man in physicochemical properties. Further, since the dog has perhaps twice the capacity of man in respect to rate of transporting oxygen to tissues, it is of interest to look for unusual adaptations in those properties of blood which relate to gas transport.

### *Composition of Serum and Cells*

The ion concentrations in serum and cells have been determined according to the same methods used by Dill, Talbott, and Edwards (3) and there are available for comparison values on nine normal men studied by them. Reference also may be made to data on electrolytes of normal human serum reported by Atchley and Benedict (4). Two specimens of dog serum and cells have been analyzed for all the quantitatively important ions. The results are compared in Table I and Table II with mean values for normal man. In dog serum there was more sodium chloride, more lactate, less bicarbonate, and less protein than in human serum. Less complete analyses of serum from other dogs also showed concentrations of sodium chloride consistently higher than in human serum. A difference in the same direction but smaller

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in amount is indicated by the results of Doisy and Beckmann (5).

Many observations indicate that the lactic acid concentration in the blood of dogs at rest is much more variable than in human blood. Values ranging from 1.5 to 4.5 milli-equivalents per liter have been found at different times in the same dog. This variation cannot be related to muscular activity for in some cases less lactic acid has been found after exercise on the treadmill than before. It is more probably related to the influence of emotional stimuli for low values are usually obtained in the case of dogs familiar with the laboratory and undisturbed by withdrawal of blood.

Analysis of other specimens of dog blood has given confirmatory evidence that there is less bicarbonate than in human blood. All the values for carbonic acid capacity of oxygenated blood given in Table III are less than the mean value for human blood; the ratio is approximately 6:7.

It has been known since Abderhalden's analyses (6) that red cells of dog blood, as contrasted with human, contain little potassium. This is in harmony with our analyses which also indicate that the total base concentration is slightly greater than in human cells and that there is slightly more chloride. The apparent large excess of anions over cations in the erythrocytes of both species is very probably due to an incorrect value for one of the anions, *viz.* proteinate. This anion is calculated according to an equation developed by Van Slyke, Wu, and McLean (7) which related pH to base bound by horse hemoglobin; evidently it gives values at least one-tenth too high for the red blood cells of dog and man.

#### *Distribution of Anion between Cells and Plasma*

It has been shown by Van Slyke, Wu, and McLean that the value of  $r$ , *i.e.*  $\frac{(\text{anion})_c}{(\text{anion})_s}$ , is determined chiefly by concentration of

non-diffusible ions in serum and in cells and by the amount of base bound by cell protein. Since these quantities have the same magnitude in the two species, the distribution of anions should be similar. Such in fact is the case. Table IV summarizes data on the blood of four dogs and of the twenty-two normal men studied by Dill, Talbott, and Edwards ((3) Table XII). There is no

TABLE I  
Acid-Base Equilibrium in Serum of Oxygenated Blood at pH<sub>s</sub> = 7.45

	Dog John	Dog Shep	Man*
H <sub>2</sub> O, cc. per l.....	946	942	939
Na, m.-eq. per l.....	149.5	146.5	139.3
K, " " "	3.4	3.7	3.3
Ca, " " "	5.4	5.2	4.7
Mg, † " " "	2.0	2.0	2.0
Σ cations, m.-eq. per l.....	160.3	157.4	149.3
Cl, m.-eq. per l.....	113.4	118.3	106.8
HCO <sub>3</sub> , m.-eq. per l.....	16.2	18.6	24.0
Proteinate, ‡ m.-eq. per l.....	15.1	16.4	17.0
HPO <sub>4</sub> <sup>=</sup> + H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> , m.-eq. per l.....	3.0	2.3	2.2
Lactate, m.-eq. per l.....	6.6	3.0	1.2
Σ anions, " " "	154.3	158.6	151.2
Protein, gm. per l.....	61.4	66.7	68.8

\* The mean values for human blood in this and the following tables are those derived by Dill, Talbott, and Edwards (3).

† The value for magnesium in serum is assumed.

‡ Proteinate of serum is calculated by the equation of Van Slyke, Hastings, Hiller, and Sendroy (8).

TABLE II  
Acid-Base Equilibrium in Cells of Oxygenated Blood at pH<sub>s</sub> = 7.45

	Dog John	Dog Shep	Man
H <sub>2</sub> O, cc. per l.....	723	736	726
Na, m.-eq. per l.....	110.3	110.5	14.9
K, " " "	4.4	6.0	93.7
Σ cations, m.-eq. per l.....	114.7	116.5	108.6
Cl, m.-eq. per l.....	57.6	59.7	51.3
HCO <sub>3</sub> , m.-eq. per l.....	9.8	11.0	12.7
Proteinate,* m.-eq. per l.....	52.4	51.8	52.6
Lactate, m.-eq. per l.....	3.4	1.6	0.8
HPO <sub>4</sub> <sup>=</sup> + H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> , m.-eq. per l.....	1.5	1.2	1.1
Σ anions, m.-eq. per l.....	124.7	125.3	118.5
Total Hb, m.-eq. per l.....	20.3	20.1	20.4

\* Proteinate of cells is calculated by the equation of Van Slyke, Wu, and McLean (7).

## Properties of Dog Blood

significant difference between the two species either in the absolute magnitude of  $r$  or in the relation of  $r_{\text{Cl}}$  to  $r_{\text{HCO}_3}$ .

TABLE III  
Buffer Value of Oxygenated Blood at 37.5°

Dog	Serum protein	HbO <sub>2</sub> capacity	CO <sub>2</sub> capacity at $p\text{CO}_2 = 40 \text{ mm.}$	$\Delta\text{CO}_2(\text{dog}-\text{man})$		
				Observed	Calculated from human blood*	Ratio
	gm. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	
John.....	52.7	8.4	18.0	5.04	5.36	0.94
A.....		10.3	17.5	6.17	5.90	1.05
".....		10.0	16.6	6.10	5.66	1.08
B.....		10.3	17.5	6.10	5.87	1.05
C.....	66.9	11.5	17.8	5.80	6.35	0.91
Shep.....	66.9	9.3	18.8	6.10	5.70	1.07
D.....	64.8	9.5	18.3	5.72	5.74	1.00
E.....	61.2	8.0	18.6	5.20	5.28	0.98
F.....	64.7	10.1	18.9	5.82	6.00	0.97
Mean.....	62.9	9.7	18.1	5.78	5.76	1.00

\* These values are calculated from the empirical chart (Fig. 3) of the paper by Henderson, Bock, Dill, and Edwards (1).

TABLE IV  
Distribution of Bicarbonate and Chloride in Oxygenated Blood at  $p\text{H}_s = 7.45$

Dog	CO <sub>2</sub> capacity at $p\text{CO}_2 = 40 \text{ mm.}$	HbO <sub>2</sub> capacity	$r_{\text{HCO}_3}$	$r_{\text{Cl}}$	$\frac{r_{\text{Cl}}}{r_{\text{HCO}_3}}$	
					m.-eq. per l.	m.-eq. per l.
John.....	18.0	8.4	0.79	0.66	0.84	
B.....	17.5	10.3	0.74	0.63	0.85	
C.....	17.8	11.5	0.78	0.66	0.85	
Shep.....	18.8	9.3	0.75	0.64	0.85	
Mean.....	18.0	9.9	0.765	0.65	0.85	
Human blood.....	21.3	9.0	0.750	0.637	0.85	

The variation of these ratios with  $p\text{H}_s$  may be represented by linear equations which, for a given anion, are nearly identical. These are as follows:

Man.....	$r_{\text{HCO}_3} = 3.52 - 0.37 \text{ pH}_s$	$r_{\text{Cl}} = 2.80 - 0.29 \text{ pH}_s$
Dog John.....	$= 3.32 - 0.34$ "	$= 2.90 - 0.30$ "
" Shep.....	$= 3.14 - 0.32$ "	$= 2.88 - 0.30$ "

### Buffer Value of Serum

A convenient expression for defining the buffer value of serum is

$$\frac{d(C_{BP_s})}{d(\text{pH}_s)} = aC_{P_s}$$

The terms are defined as given by Henderson, Dill, Edwards, and Morgan (2); *viz.*

$C_{BP_s}$  = milli-equivalents of base bound by serum protein per liter of serum  
 $C_{P_s}$  = gm. of serum protein per liter of serum

Comparison may be made conveniently over the extreme physiological range,  $\text{pH}_s = 7.0$  to  $\text{pH}_s = 7.6$ .

The factor  $a$  measures the slope of the serum buffer curve per unit of protein. Since about 98 per cent of the buffering in normal human serum, separated from cells, is due to serum protein, it follows that  $a$  is an approximate measure of buffer value of serum protein. Van Slyke, Hastings, Hiller, and Sendroy (8) have shown that albumin and globulin of serum have different buffer values ( $a_{\text{alb.}} = 0.125$ ;  $a_{\text{glob.}} = 0.076$ ) and hence the value of  $a$  depends upon the proportion of the two proteins present. The data of Henderson, Dill, Edwards, and Morgan (2) indicate that  $a$  is quite constant for normal human serum. Its mean value (see their Table I, Column 6) over the  $\text{pH}_s$  range in question is 0.110. It seems to be less constant in dogs, for six experiments have given the values 0.082, 0.085, 0.094, 0.097, 0.097, and 0.102. The mean, 0.093, is one-sixth less than for human serum. The facts suggest a greater and more variable proportion of globulin in dog serum than in human serum but it cannot be assumed that the inherent properties of the individual proteins are identical in the two species.

### Buffer Value of Oxygenated Blood

Henderson and his associates (1) have described the slope of the carbonic acid dissociation curve of oxygenated human blood as a function of carbonic acid capacity and hemoglobin content.

Their empirical chart makes possible a comparison of the buffer value of dog blood and of human blood of the same carbonic acid capacity and hemoglobin content. Such a comparison, made in Table III, shows that the buffer value of oxygenated dog blood equals very nearly that of human blood.

On the basis of this observation and the facts (*a*) the buffer value of dog serum per unit of protein is slightly less than that of human serum and (*b*) the protein content of dog serum is slightly less than that of human serum, it follows that the buffer value of oxygenated red blood cells is greater, possibly by one-twentieth, in the dog than in man. Since the principal buffer of cells is hemoglobin, dog hemoglobin must closely resemble human hemoglobin in buffer value. This conclusion is interesting in view of the comparison made by Hastings, Van Slyke, Neill, Heidelberger, and Harington (9) of dog oxyhemoglobin with horse oxyhemoglobin. Their results indicate that the former has about four-fifths the buffer value of the latter at constant cation concentration and at pH values ranging from 6.9 to 7.3.

#### *Oxygen Dissociation Curves*

The method used in deriving oxygen dissociation curves such as those shown in Fig. 1 is as follows:

Experimental values are obtained for  $pCO_2$ ,  $pO_2$ , and  $HbO_2$  for each of twelve to twenty points. In addition one calculates by interpolation values for total  $CO_2$  of blood and plasma for each of these points. Such a purpose is served by carbonic acid dissociation curves of oxygenated blood, of reduced blood, and of the corresponding true plasma, portions of the same specimen of blood being used for all points. Observations on cell volumes are required in order to calculate the total  $CO_2$  content of cells corresponding to each point on the oxygen dissociation curves. Free  $CO_2$  is calculated from values for  $pCO_2$  and the solubility coefficient for cells established by Van Slyke, Sendroy, Hastings, and Neill (10). In order to calculate  $pH_c$ , one must know  $pK_c'$ . This quantity was evaluated for mammalian cells and its dependence on  $HbO_2$  established by Stadie and Hawes (11). Finally one obtains by the Henderson equation  $pH_c$  values for each point on the oxygen dissociation curves. If the  $pCO_2$  values have been properly chosen, sets of values for  $pH_c$  are obtained and a family

of oxygen dissociation curves can be drawn, one curve for each  $pH_e$  value. If desired, the curves thus obtained can be transformed to a common curve making use of the well known fact that members of a family of oxygen dissociation curves are parallel when  $\log pO_2$  is plotted as a function of  $\log \frac{Hb}{HbO_2}$ . That is, for a given increment in pH there is for all values of  $HbO_2$  a constant increment in  $\log pO_2$ . It is also useful that, in the physiological

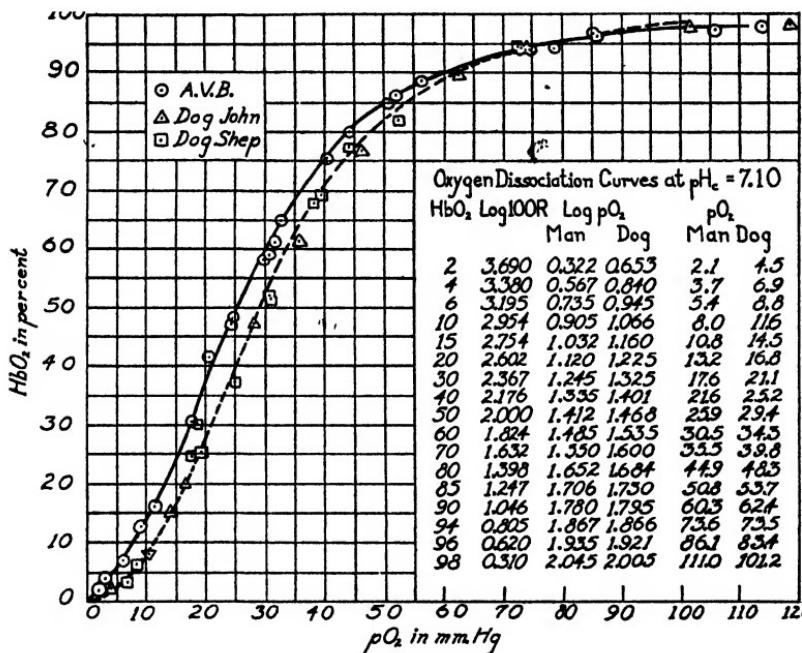


FIG. 1. Oxygen dissociation curves of the blood of man (A. V. B.) and of two dogs at an equilibration temperature of 37.5° and when  $pH_e = 7.1$ .

range, there is a linear relation between pH and  $\log pO_2$ ,  $HbO_2$  remaining constant. This will be referred to later.

The results of such experiments and calculations are embodied in the two curves shown in Fig. 1. By coincidence, specimens of the blood of the two dogs had a common oxygen dissociation curve at the same hydrogen ion concentration. The difference between this common curve and that of A. V. B. is well outside experimental

error but not greater than one finds between individuals of either species. The range in position of the normal oxygen dissociation curve has been determined for eight normal men by Dill, Edwards, Fölling, and others ((12) Table I). At one-half saturation and when  $pH_c = 7.1$ ,  $pO_2$  values ranged from 25.6 to 28.2. In the present experiments the  $pO_2$  values at one-half saturation are 25.9 for A. V. B. and 29.4 for the two dogs; *viz.*, the blood of A. V. B. is near the lower limit among normal men while that of the two dogs is near the upper limit for normal men. Specimens of blood from two other dogs were examined in order to gain some idea of the extent of variation among individuals of this species. The position of the curve expressed as above, was 24.2 for one and 32.7 for the other. These facts are of interest in view of the suggestion once advanced by Barcroft and Camis (13) that the ratio of sodium to potassium is of significance in determining the form and position of the oxygen dissociation curve. In view of the extreme difference between the two species in one respect and the close similarity in the other, there is probably no relation between this ratio and the nature of the curves.

#### *Acid Properties of Reduced and Oxygenated Hemoglobin*

This subject has been investigated by Hastings, Van Slyke, Neill, Heidelberger, and Harington (9). They found that in dog hemoglobin the maximum value for  $\frac{d(BHb)}{d(HbO_2)}$  was about nine-tenths the maximum in horse hemoglobin. So far as our observations go, no consistent difference was found in this respect between man and dog. Probably the most sensitive test of this question consists in a study of the reciprocal effect; *viz.*, that of acid change upon the affinity of blood for oxygen. Such a comparison is made in Fig. 2 and it is evident that the slopes of the curves relating  $\log pO_2$  at half saturation to  $pH_c$  are similar, if not identical, in man and dog. The relation for dog blood in physiological limits is

$$\log pO_2 = 5.24 - 0.53 \text{ pH}_c$$

#### *Miscellaneous Observations*

The reaction of arterial dog blood was calculated in ten cases from its carbonic acid content and capacity. In eight of these

cases the pH of arterial serum in rest<sup>1</sup> varied from 7.33 to 7.39 and averaged 7.38. The arterial carbonic acid pressure in the same eight specimens ranged from 37.7 to 44.4, averaging 40.9.<sup>1</sup> Thus arterial dog blood has the same carbonic acid pressure as in man and is slightly more acid than in man. These facts harmonize with the observation that the carbonic acid capacity is less than in man. The rectal temperatures in rest had a mean value of 38.9°, a minimum of 37.9°, and a maximum of 39.9°.

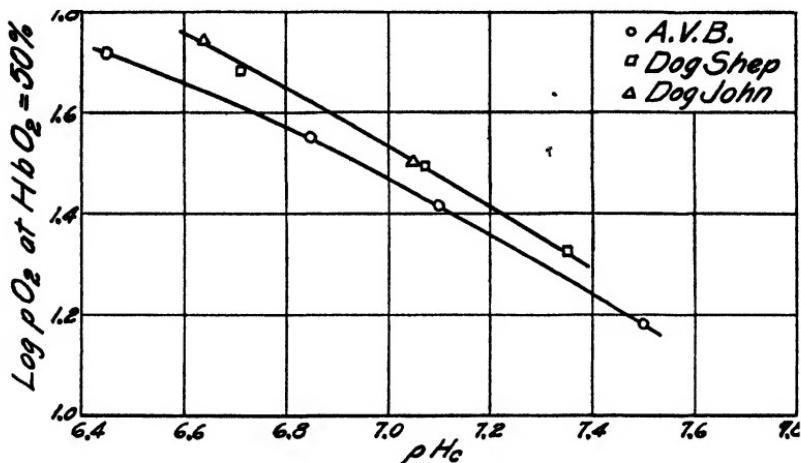


FIG. 2. Position of the oxygen dissociation curves ( $\log pO_2$  when  $HbO_2 = 50$  per cent) in relation to change in  $pH_c$ .

#### SUMMARY

The buffer value of dog serum per unit of protein is about five-sixths that of human serum. The buffer value of dog blood is the same as that of human blood and it follows that dog hemoglobin and human hemoglobin have nearly the same buffer value.

The distribution of chloride and bicarbonate between cells and plasma is not significantly different in these species. In each case in oxygenated blood at  $pH_c = 7.45$ ,  $r_{Cl} = 0.64 = 0.85r_{HCO}$ , and the effect of change in hydrogen ion concentration on these ratios is also the same for the two species.

<sup>1</sup> In the calculation of  $pH_c$ , account was taken of the temperature in each case and of its effect upon  $pK'$  and on carbon dioxide solubility.

Dog blood usually has from 4 to 8 per cent greater chloride content than human blood and the ratio of sodium to potassium in cells is very large.

The oxygen dissociation curve of dog blood is little different from that of human blood and the effect of acid upon its position is the same.

The carbonic acid pressure of arterial blood is about the same in man and the dog but the blood of the latter is slightly more acid and has a slightly lower carbonic acid capacity.

In general there appears to be close similarity between dog blood and human blood. The greater capacity of the dog for transporting oxygen from lungs to tissues cannot be related to the properties of his blood.

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## ON WALDEN INVERSION

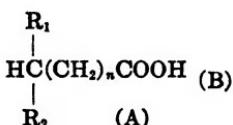
### XVII. OPTICAL ROTATIONS IN HOMOLOGOUS SERIES OF CARBOXYLIC ACIDS

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New York)

(Received for publication, December 3, 1931)

The present communication deals with the optical rotation of a series of disubstituted carbonic acids of the type

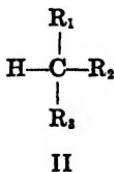


Group  $\text{R}_1$  was kept constant in the entire series of substances examined. Group  $\text{R}_2$  was a normal alkyl radicle which was progressively increased in weight. The subscript  $n$  remained constant in every vertical series and increased progressively in the horizontal series. Thus, series were obtained in which the members changed with respect to one group only. In Table I the vertical columns represent a homologous series with respect to group  $\text{R}_2$ ; the horizontal rows, a series homologous with respect to the group— $(\text{CH}_2)\text{COOH}$ .

In previous communications,<sup>1</sup> observations were reported permitting of the conclusion that the optical rotation of each substance may be regarded as if it were the resultant of two major contributions. The data presented in the present communication deal with the quantitative changes in the rotations of series homologous to one radicle only, thus showing the effect on the total rotation of the change in value of a single contribution.

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77, 405, 687, 761 (1931); **92**, 435 (1931); **93**, 749 (1931); **95**, 1 (1932).

In the case of normal hydrocarbons of the type of trisubstituted methanes, the two heavier groups should furnish contributions of opposite sign. Thus in the series



an increase in weight of  $R_3$  should bring about an increase of the molecular rotation towards the right whereas an increase in weight of  $R_2$  should have the opposite effect. The observations reported in previous communications are in harmony with this expectation. When neither  $R_2$  nor  $R_3$  is a simple alkyl radicle, a prediction is impossible since the contribution of any group depends upon its structure and consequently it may function as a lighter or as a heavier group than an alkyl group of the same weight. However, taking a single substance (II) and forming from it two homologous series, one with respect to  $R_3$  and the other with respect to  $R_2$ , it may be possible to arrive at a conclusion as to the sign of the contribution of each group and also as to the relative value of each group. Thus, if  $[M] = A + B$  and if  $A$  has a higher value than  $B$ , the direction of the rotation is determined by that of the contribution  $A$ . Then the values of the rotations of the consecutive members of the series homologous with respect to  $A$  should progressively increase. If the value of  $B$  is greater than  $A$ , the direction of rotation is determined by that of the contribution  $B$ . In this case, if a series is formed homologous to  $A$ , the changes in the rotations of the members of the homologous series should be in the direction of  $A$ , although the values may progressively diminish. Thus, if  $A$  is positive and  $B$  is negative and of a higher value than  $A$ , the substance should rotate to the left but as  $A$  progressively increases in weight,  $B$  remaining approximately constant, the values of the negative rotations of the successive members of the homologous series should progressively decrease, thus indicating a change towards the right.

The theoretical implications here described are illustrated in Table I. In Table I the values of the rotations given for the

TABLE I  
Maximum Molecular Rotations of Configurationally Related Aliphatic Acids Containing a Methyl Group on the Asymmetric Carbon Atom [ $M$ ]<sup>a</sup>

(1)	(2)	(3)	(4)	(5)
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$
$\text{C}_2\text{H}_5$				
- 18.0*	- 10.4	- 13.6	- 11.1	- 12.2
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HO}-\text{COOH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \end{array}$
$\text{C}_2\text{H}_5 \text{ (n)}$	+ 3.6	- 6.9	- 3.7	
Levo				
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \text{ (n)} \end{array}$
$\text{C}_2\text{H}_5 \text{ (n)}$	+ 6.1	- 4.1	- 1.7	
Levo				
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HO}-\text{COOH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_{11} \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_{11} \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_{11} \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_{11} \text{ (n)} \end{array}$
$\text{C}_2\text{H}_{11} \text{ (n)}$	+ 8.1	- 1.9	- 0.6	
Levo				

\* Marekwald, W., Ber. chem. Ges., **37**, 1045, 1048 (1904).

members of the first vertical series are not the maximum values whereas those of all other members are maximum values. A comparative analysis of the second vertical series may serve as a key to the character of each of the two contributions in all the substances presented in Table I. The rotation of the first member is negative and that of all successive members is increasingly positive, indicating that the contribution of the heavier alkyl radicle is positive and that of the group containing the carboxyl is negative. In the first member the direction of rotation is determined by the  $-(CH_2)COOH$  group; in the remaining members by that of the heavier alkyl radicle. Assuming then that the direction of the contribution A remains constant for all these configurationally related substances, the conclusion is reached that in all the substances enumerated in Table I the contribution B is levorotatory. In the substances of Column 1, the value of B is higher than that of A, hence all the members rotate to the left. In Column 2 the negative value of B is smaller than in Column 1 so that it determines the direction of rotation of the first member only. In Column 3 the value of B is higher than A, so that all members rotate to the left but the rotations progressively change towards the right because of the increase in the dextrorotation of the contribution A. Finally, in Column 4 the value of B is still higher than that of A but not to the same extent as for the members of Column 3; hence the values of rotation of the individual members are lower than those of the corresponding members of Column 3 and again the change of rotation of the individual members is progressively towards the right.

Comparing the members of any *horizontal row*, one observes an alternating change in the values of the rotations of individual members. Thus it seems as if the carboxyl group attached directly to the asymmetric carbon atom furnishes a higher negative contribution than the corresponding group of the second horizontal member. The third member furnishes a higher contribution than the second and a lower than the fourth, thus the contributions are  $1 > 2 < 3 > 4$ . Thus, the effect of distance upon the contribution of a polar group is to change the value only and not the sign.

In Table II are given the maximum rotations of certain derivatives of the disubstituted propionic acids; namely, the disubstituted ethylcarbinols and disubstituted propyl bromides. In the series of these substances given in Columns 2, 3, and 4, the values of the rotations increase progressively to the right. On the basis of these observations it is possible to predict that in every other homologous series derived from the radicles given in Column 1, the values will progressively increase towards the right. Now the

TABLE II  
*Effect on Rotation of Polarity of Substituting Groups [M]<sub>D</sub>*<sup>25</sup>

(1)	—CH <sub>2</sub> COOH (2)	—CH <sub>2</sub> CH <sub>2</sub> OH (3)	—CH <sub>2</sub> CH <sub>2</sub> Br (4)	(5)
CH <sub>3</sub> HC— C <sub>2</sub> H <sub>5</sub>	—10.4	—9.2	—27.8	CH <sub>3</sub> HC—OH +10.3 C <sub>2</sub> H <sub>5</sub>
CH <sub>3</sub> HC— C <sub>3</sub> H <sub>7</sub> , (n)	+3.6	+2.1	—21.3	CH <sub>3</sub> HC—OH +12.1 C <sub>3</sub> H <sub>7</sub> , (n)
CH <sub>3</sub> HC— C <sub>4</sub> H <sub>9</sub> , (n)	+6.1	+4.0	—16.8	CH <sub>3</sub> HC—OH +11.8 C <sub>4</sub> H <sub>9</sub> , (n)
CH <sub>3</sub> HC— C <sub>6</sub> H <sub>11</sub> , (n)	+8.0	+6.1	—14.7	CH <sub>3</sub> HC—OH +12.0 C <sub>6</sub> H <sub>11</sub> , (n)

dextrorotatory secondary carbinols of the methyl series are known to possess the configuration given in Column 5. The first member of this series has a lower dextrorotation than the succeeding members. It is assumed that they are configurationally related to the other substances given in Tables I and II. On the basis of these considerations, the configuration given in Column 1 of Tables I and II was assigned as well to all the other substances given in these tables.

Thus the concept that the rotation of a given substance is

influenced by two contributions furnishes a method by which the configurations of two substances can be correlated provided there are available or are obtainable data regarding the maximum rotations of the two respective homologous series to which the substances belong. Hence it should be possible to correlate the halides derived from the radicles given in Column 1 if data on the maximum rotations of the members of the homologous series were available.

TABLE III  
*Correlation of Methyl-n-Butylpropionic Acid with Methyl-n-Butylacetic Acid*

$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_4\text{H}_9(n) \\ -5.0^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COCl} \\   \\ \text{C}_4\text{H}_9(n) \\ +2.9^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CONH}_2 \\   \\ \text{C}_4\text{H}_9(n) \\ (\text{Levo})^* \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{NH} \\   \\ \text{C}_4\text{H}_9(n) \\ -13.5^\circ \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_4\text{H}_9(n) \\ +6.3^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COCl} \\   \\ \text{C}_4\text{H}_9(n) \\ +7.5^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CONH}_2 \\   \\ \text{C}_4\text{H}_9(n) \\ (\text{Dextr})^* \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CN} \\   \\ \text{C}_4\text{H}_9(n) \\ +10.4^\circ \end{array}$
$\nearrow$ $\swarrow$			

\* In alcohol.

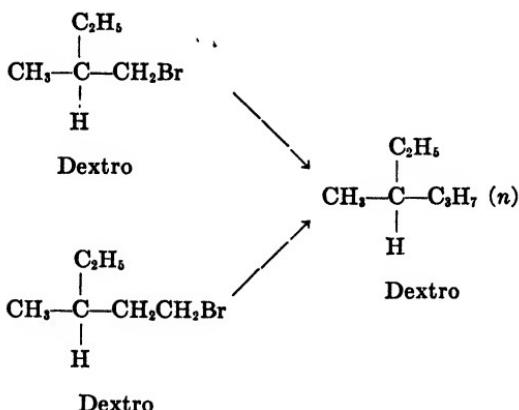
It is desired to emphasize again that the contribution of each group is not an independent property characteristic for any particular group, but is the resultant of the vicinal effect of all other groups; hence the contributions of the invariable groups in each homologous series are not absolutely constant. However, their values change in a degree which is minor as compared with that of the change introduced by the variable group.

*Preparation of the Series of Disubstituted Carbonic Acids*

The starting materials for the acids given in Table I were the corresponding disubstituted propionic acids given in Column 2 of that table. These acids have been resolved to the maximum rotations. They were converted into the corresponding carbinols which were, in turn, converted into the bromides. The maximum rotations of the derived substances were calculated on the basis of the maximum rotations of the disubstituted propionic acids.

*Configurational Relationship of Members of the Series of Disubstituted Acetic and Propionic Acids*

The configurational relationship existing between the higher members of the horizontal series derived from the 1,1-disubstituted propionic acids is self-evident and needs no further discussion. The configuration of methylethylacetic acid has been correlated previously with that of 1-methyl-1-ethylpropionic acid-3 on the basis of the following reaction.



On the basis of the set of reactions given in Table III, the same conclusion has now been reached for the higher members.

**EXPERIMENTAL**

*Levo-2-n-Propylhexanoic Acid*-6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 40 gm. of 1-bromo-3-methylhexane,  $[\text{M}]_D^{25} = -3.17^\circ$ . The product was refluxed with stirring for 3

hours, poured into water, and the ester extracted with ether. The ether was distilled off and the ester hydrolyzed by boiling with 60 gm. of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and water added. The aqueous solution was acidified with hydrochloric acid and extracted with ether. The ether was evaporated and the residue heated under atmospheric pressure at 180° in a metal bath until carbon dioxide ceased to be evolved. The acid was then distilled and purified through

its sodium salt. B.p., 127° at 5 mm.; yield, 24 gm.;  $D \frac{25}{4} = 0.901$ .

$$[\alpha]_D^{25} = \frac{-0.66^\circ}{2 \times 0.901} = -0.37^\circ. [M]_D^{25} = -0.58^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -3.67^\circ$  (homogeneous).

3.316 mg. substance: 8.320 mg. CO<sub>2</sub> and 3.370 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 68.3, H 11.5

Found. " 68.4, " 11.4

*Levo-2-n-Butylhexanoic Acid-6*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 32 gm. of 1-bromo-3-methylheptane,  $[M]_D^{25} = -9.01^\circ$ . The acid was isolated and purified as described for 2-propylhexanoic acid-6. B.p., 130° at 3 mm.; yield, 22 gm.;

$D \frac{25}{4} = 0.897$ .

$$[\alpha]_D^{25} = \frac{-0.90^\circ}{2 \times 0.897} = -0.50^\circ. [M]_D^{25} = -0.86^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -1.6^\circ$  (homogeneous).

4.944 mg. substance: 12.685 mg. CO<sub>2</sub> and 5.275 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>O<sub>2</sub>. Calculated. C 69.7, H 11.7

Found. " 69.9, " 11.9

*Levo-2-n-Amylhexanoic Acid-6*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 34 gm. of 1-bromo-3-methyloctane,  $[M]_D^{25} = -8.30^\circ$ . The acid was prepared and purified as described for 2-propylhexanoic acid-6. B.p., 135° at 3 mm.; yield, 26 gm.;

$D \frac{25}{4} = 0.893$ .

$$[\alpha]_D^{\infty} = \frac{-0.32^\circ}{2 \times 0.893} = -0.18^\circ. [M]_D^{\infty} = -0.33^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -0.6^\circ$  (homogeneous).

4.180 mg. substance: 10.835 mg. CO<sub>2</sub> and 4.480 mg. H<sub>2</sub>O  
 $C_{11}H_{22}O_2$ . Calculated. C 70.9, H 11.9  
 Found. " 70.7, " 12.0

*Dextro-3-Methylhexanoic Acid-6*—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 40 gm. of 1-chloro-3-methylpentane,  $[M]_D^{25} = +6.51^\circ$ . Carbon dioxide was passed into the cooled solution for 30 minutes. The Grignard solution was decomposed by pouring onto ice and hydrochloric acid. The organic acid was extracted with ether, and then purified through its sodium salt. B.p., 115° at 16 mm.; yield, 29 gm.;

$$D \frac{22}{4} = 0.923.$$

$$[\alpha]_D^{\infty} = \frac{+2.85^\circ}{1 \times 0.923} = +3.09^\circ. [M]_D^{\infty} = +4.02^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{22} = +13.6^\circ$  (homogeneous).

2.848 mg. substance: 6.700 mg. CO<sub>2</sub> and 2.838 mg. H<sub>2</sub>O  
 $C_7H_{14}O_2$ . Calculated. C 64.6, H 10.8  
 Found. " 64.2, " 11.1

*Dextro-4-Methylheptanoic Acid-7*—This acid was prepared by passing carbon dioxide into a Grignard reagent formed from 45 gm. of 1-bromo-3-methylhexane,  $[M]_D^{21} = +9.29^\circ$ , and 6 gm. of magnesium. It was purified as described for 3-methylhexanoic acid-6. B.p., 132° at 22 mm.; yield, 31 gm.; D  $\frac{24}{4} = 0.882$ .

$$[\alpha]_D^{\infty} = \frac{+1.86^\circ}{1 \times 0.882} = +2.11^\circ. [M]_D^{\infty} = +3.04^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{24} = +6.9^\circ$  (homogeneous).

3.475 mg. substance: 8.525 mg. CO<sub>2</sub> and 3.500 mg. H<sub>2</sub>O  
 $C_8H_{16}O_2$ . Calculated. C 66.6, H 11.2  
 Found. " 66.9, " 11.3

*Levo-5-Methyloctanoic Acid-8*—The acid was prepared by passing carbon dioxide into a Grignard reagent formed from 50 gm. of 1-bromo-3-methylheptane,  $[M]_D^{24} = -9.01^\circ$ , and 6 gm. of magnesium in ether. The acid was extracted and purified as described for 3-methylhexanoic acid-6. B.p.,  $149^\circ$  at 22 mm.; yield, 33 gm.;

$$D \frac{25}{4} = 0.871.$$

$$[\alpha]_D^{\infty} = \frac{-1.17^\circ}{1 \times 0.871} = -1.33^\circ. [M]_D^{\infty} = -2.10^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -4.1^\circ$  (homogeneous).

3.191 mg. substance: 7.935 mg. CO<sub>2</sub> and 3.320 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 68.3, H 11.7

Found. " 67.8, " 11.6

*Levo-6-Methylnonanoic Acid-9*—This acid was prepared by passing carbon dioxide into a Grignard reagent formed from 52 gm. of 1-bromo-3-methyloctane,  $[M]_D^{24} = -8.30^\circ$ . The acid was isolated and purified as described for 3-methylhexanoic acid-6.

$$\text{B.p., } 156^\circ \text{ at 22 mm.; yield, 37 gm.; } D \frac{25}{4} = 0.871.$$

$$[\alpha]_D^{\infty} = \frac{-0.52^\circ}{1 \times 0.871} = -0.59^\circ. [M]_D^{\infty} = -1.01^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -1.9^\circ$  (homogeneous).

3.975 mg. substance: 10.135 mg. CO<sub>2</sub> and 4.145 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>22</sub>O<sub>2</sub>. Calculated. C 69.7, H 11.7

Found. " 69.5, " 11.7

*Dextro-3-Methylheptanoic Acid-7*—A Grignard reagent was prepared from 4 gm. of magnesium in dry ether and 30 gm. of 1-bromo-4-methylhexane,  $[M]_D^{25} = +4.67^\circ$ . Dry carbon dioxide was passed for 15 minutes into the cooled Grignard solution. The product was decomposed by means of ice and the acid purified through its sodium salt. B.p.,  $128^\circ$  at 20 mm.; yield, 12 gm.;

$$D \frac{26}{4} = 0.893.$$

$$[\alpha]_D^{\infty} = \frac{+2.21^\circ}{1 \times 0.893} = +2.47^\circ. [M]_D^{\infty} = +3.56^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = +11.07^\circ$  (homogeneous).

5.220 mg. substance: 12.765 mg. CO<sub>2</sub> and 5.150 mg. H<sub>2</sub>O  
 $C_8H_{16}O_2$ . Calculated. C 66.6, H 11.2  
 Found. " 66.7, " 11.0

*Dextro-3-Methyloctanoic Acid-8*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 30 gm. of 4-methyl-1-bromohexane,  $[M]_D^{25} = +4.67^\circ$ . The product was boiled with stirring for 2 hours, then the ester was isolated and hydrolyzed with potassium hydroxide. The salt was extracted with ether, then acidified, and the substituted malonic acid extracted with ether. The ether was distilled off and the acid heated to 190° in a metal bath until carbon dioxide ceased to be evolved. It was then distilled. B.p., 139° at 20 mm.; yield, 18 gm.; D  $\frac{25}{4} = 0.899$ .

$$[\alpha]_D^{25} = \frac{+2.24^\circ}{1 \times 0.899} = +2.49^\circ. [M]_D^{25} = +3.93^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = +12.22^\circ$  (homogeneous).

4.853 mg. substance: 12.139 mg. CO<sub>2</sub> and 5.005 mg. H<sub>2</sub>O  
 $C_8H_{16}O_2$ . Calculated. C 68.3, H 11.5  
 Found. " 68.2, " 11.5

*Levo-1-Amino-2-Methylhexane*—50 gm. of 2-butylbutyric acid-4,  $[\alpha]_D^{25} = -3.46^\circ$  (homogeneous), were refluxed on a steam bath for 15 minutes with an excess of thionyl chloride. The excess thionyl chloride was then distilled off and the residue dropped slowly into 200 cc. of cold aqueous ammonium hydroxide. The amide was filtered off, recrystallized from 50 per cent alcohol, and dried in a vacuum desiccator over sulfuric acid.

The amide was mixed with 55 gm. of bromine (1 mol) and the mixture poured into 500 cc. of 20 per cent potassium hydroxide previously heated to 70°. It was allowed to stand on a steam bath 2 hours, cooled, and the amine extracted with ether. The ether extract was shaken with dilute hydrochloric acid and then the aqueous layer was extracted several times with ether to remove impurities. The aqueous layer was made alkaline with potassium hydroxide solution and the amine was extracted with ether, dried

with sodium sulfate, and distilled. B.p., 62° at 22 mm.; yield,  
11 gm.; D  $\frac{27}{4}$  = 0.773.

$$[\alpha]_D^{\text{''}} = \frac{-9.08^\circ}{1 \times 0.773} = -11.75^\circ. \quad [M]_D^{\text{''}} = -13.51^\circ \text{ (homogeneous)}$$

4.465 mg. substance: 11.863 mg. CO<sub>2</sub> and 5.860 mg. H<sub>2</sub>O  
C<sub>7</sub>H<sub>17</sub>N. Calculated. C 72.9, H 14.8  
Found. " 72.5, " 14.7

## A METHOD FOR THE DETERMINATION OF THYROXINE IN THE THYROID\*

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Since it is now known (1, 2) that the thyroid gland contains at least one iodine compound other than thyroxine, it has become important to know how much of the iodine of the gland is in the form of the physiologically active substance, thyroxine. A method for the estimation of thyroxine would be useful in assaying therapeutic preparations as well as in studies of thyroid physiology and pathology. Harington and Randall (3) have proposed a method for this purpose which is extremely simple technically, being merely a determination of the total iodine in the acid-insoluble fraction after partial hydrolysis of the gland with sodium hydroxide.<sup>1</sup> Although their procedure undoubtedly allows a more satisfactory assay of thyroid preparations than was hitherto possible, nevertheless we feel that it is not sufficiently accurate for certain purposes. Our evidence for this belief will be given in this paper together with the details of a method which appears to determine more accurately the thyroxine in thyroid material.

The method is based on the selective extraction of thyroxine by butyl alcohol after alkaline hydrolysis of the gland. Distribution ratios between butyl alcohol and 2 N sodium hydroxide were determined for thyroxine, diiodotyrosine, and inorganic iodide, the three iodine compounds known to be present in an alkaline hydrolysate of thyroid gland. The results which are shown in Table I

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† The data in this paper are taken from a thesis submitted by Jessica P. Leland in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

<sup>1</sup> See also the earlier work of Wilson and Kendall (4).

were obtained at room temperature. Determinations made at 0° were found to be practically identical, hence ordinary temperature variations are not important. Furthermore, the ratios are practically the same from both N and 2 N sodium hydroxide. The effect of concentration of the iodine compounds on the ratios was determined in the case of diiodotyrosine and thyroxine where a 10-fold increase in concentration made little or no change in the ratios. In the case of potassium iodide the actual concentrations dealt with in the method to be described are so small that it seems unlikely that the distribution ratio would vary within the ranges of concentration encountered.

Any possible effect on the distribution ratios due to presence of the products of protein hydrolysis was ruled out by determining

TABLE I  
*Distribution Ratios of Thyroxine, Diiodotyrosine, and Potassium Iodide between Butyl Alcohol and 2 N NaOH*

Substance	Ratio concentration in BuOH concentration in NaOH	Approximate concentration at equilibrium
Thyroxine.....	92:8	0.0003 M in BuOH
“ .....	95:5	0.003 “ “ “
Diiodotyrosine.....	2.5:97.5	0.004 “ “ NaOH
“ .....	2.0:98.0	0.04 “ “ “
KI.....	7.6:92.4	0.007 “ “ “

the ratios for distribution of diiodotyrosine and thyroxine between butyl alcohol and the solution obtained on hydrolyzing 2 gm. of casein in 100 cc. of 2 N NaOH for 18 hours. Ratios of 2.2:97.8 and 94.5:5.5 were found for diiodotyrosine and thyroxine respectively, values which are not significantly different from those in Table I.

With these data, it was calculated that 99.0 per cent of the thyroxine, 0.27 per cent of the diiodotyrosine, and 1.64 per cent of the inorganic iodide would remain in the butyl alcohol layer after the following program of extraction.

Shake the 2 N sodium hydroxide solution containing the iodine compounds with an equal volume of butyl alcohol. Separate. Shake the aqueous layer again with a second equal volume of butyl alcohol. Separate. Combine the butyl alcohol extracts and wash

them with an equal volume of N NaOH. Separate. Extract the sodium hydroxide washings with 0.5 volume of butyl alcohol. Combine the butyl alcohol fractions.

To compare theory with practice, known amounts of the pure substances dissolved in 100 cc. of 2 N NaOH were subjected to the above schedule of extraction and washing and the final butyl alcohol fractions ashed for determination of iodine. The results, shown in Table II, are in good agreement with the theory. The fact that an appreciable fraction of the inorganic iodide remains in the butyl alcohol does not seriously affect the thyroxine determination, for the total amount of iodide in a hydrolysate is small and the fraction of it which remains in the butyl alcohol is negligible.

TABLE II  
*Observed and Calculated Recoveries after Butyl Alcohol Extraction*

Substance	Amount of I <sub>2</sub> taken	Expected on basis of distribution ratio		Found by analysis	
		mg.	per cent	mg.	per cent
Thyroxine	1.065	1.058	99.0	1.039	97.6
Diiodotyrosine	3.453	0.007	0.19	0.010	0.29
KI	4.689	0.077	1.65	0.082	1.68
Thyroxine in presence of 8 times as much iodine in form of diiodotyrosine	0.431 as thyroxine 3.453 as diiodotyrosine	0.433		0.425	$\frac{0.425}{0.431} = 98.6$

#### *Hydrolysis of Thyroid Gland*

The proposed method postulates hydrolysis of the thyroid sufficiently complete that the thyroxine shall be free from its combination in the gland. Since thyroxine is slowly destroyed by heating with alkali, it was necessary to find conditions which would give complete hydrolysis of the protein with minimum destruction of thyroxine. It was necessary to determine the most suitable conditions as to concentration of alkali, concentration of protein being hydrolyzed, and time of hydrolysis. The material used in this preliminary work was from a large and uniform lot of commercial desiccated thyroid. After hydrolysis under the various conditions, the solutions were extracted according to the scheme

mentioned above, and the final butyl alcohol extracts containing the thyroxine were ashed for determination of total iodine. The results of this preliminary search for the optimum conditions of hydrolysis are shown in Fig. 1.

Increasing yields of thyroxine were obtained up to 18 hours after which no further gain was apparent. Hydrolysis may be regarded as complete, therefore, at 18 hours but not before that time. Over longer periods of time the destruction of free thyroxine appears to be very slow.

The effect of different concentrations of alkali was found by hydrolyzing 2.5 gm. of desiccated thyroid with 100 cc. of N, 2 N, and 4 N NaOH for 18 hours. The yields of thyroxine in terms of percentage of total iodine were respectively 16.8, 21.3, and 21.7.

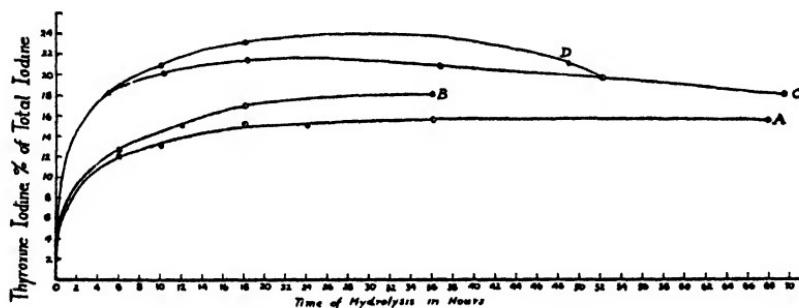


FIG. 1. Curves showing yields of iodine in thyroxine fraction under different conditions and times of hydrolysis. Curve A, 5 gm. of thyroid (Burroughs Wellcome) hydrolyzed with 100 cc. of N NaOH; Curve B, 2.5 gm. hydrolyzed with 100 cc. of N NaOH; Curve C, 2.5 gm. hydrolyzed with 100 cc. of 2 N NaOH; Curve D, 1.25 gm. hydrolyzed with 100 cc. of 2 N NaOH.

2 N alkali is thus shown to be a more effective hydrolyzing agent than N alkali. No significant further increase is apparent when 4 N alkali is used.

The yield of thyroxine varies somewhat with the proportion of gland substance to volume of alkali used in the hydrolysis. With 100 cc. of 2 N NaOH and 5, 2.5, 1.25, and 0.75 gm. of thyroid, the yields of iodine in the butyl alcohol fraction were respectively 20.6, 21.3, 23.1, and 22.8 per cent of the total iodine of the gland. The proportion 1.25 gm. to 100 cc. of 2 N alkali was chosen as the most suitable.

Attempts were made to recover added thyroxine under varying conditions. In each case a known amount (about 1.5 mg.) of pure thyroxine dissolved in 0.1 N NaOH was added to the charge of thyroid substance before hydrolysis.

As shown in Table III the best recovery of added thyroxine was obtained when 2 N alkali was used and the proportion either of 2.5 or 1.25 gm. of thyroid material to 100 cc. of alkali. In an effort to discover why we were able to recover only 83 per cent, a study was made of the effect of boiling alkali on pure thyroxine (see Table IV).

TABLE III  
*Recovery of Added Thyroxine*

Desiccated thyroid	NaOH, 100 cc.	Added thyroxine recovered
gm.	N	per cent
5	1	69.0
2.5	1	75.8
2.5	2	83.3
1.25	2	83

TABLE IV  
*Effect of Boiling Alkali on Pure Thyroxine*

Time of boiling	NaOH	Recovery of thyroxine
hrs.	N	per cent
18	1	95.6
63	1	60.0
18	2	74.6

2 N alkali is seen to be much more destructive to pure thyroxine than N alkali, the recovery being only 74.6 per cent in one case, while it is 95.6 per cent in the other. This, without doubt, accounts for the recovery of only 83 per cent of thyroxine when added to desiccated thyroid and hydrolyzed for 18 hours. We cannot explain why the recovery of added thyroxine when N alkali is used for hydrolyzing the gland is less than that found when 2 N is used (Table III); whereas in Table IV 2 N alkali is shown to be the more destructive to pure thyroxine. To determine whether the 8 per cent difference between the recovery of thyroxine alone and in the presence of gland material was due to some protective action given by the protein itself, thyroxine was added to casein which

was then boiled with alkali. When pure thyroxine was added to 1.25 gm. of Hammarsten casein and hydrolyzed for 18 hours with 100 cc. of 2 N NaOH, a recovery of 81 per cent was obtained.

Recovery of thyroxine after boiling 18 hours with 2 N sulfuric acid was less favorable than when alkali was used, only 62 per cent being recovered.

From the curves on Fig. 1 it can be seen that to obtain the highest yield of thyroxine 2 N NaOH must be used as the hydrolytic agent in spite of the fact that serious destruction of the freed thyroxine is indicated from our work with the pure substance. While we are unable to furnish direct evidence that the method which we propose will give us more than 83 per cent of the thyroxine originally present, since we are unable to recover more than 83 per cent of the pure substance when added to thyroid material, we believe that destruction is not nearly so great of the thyroxine built into the protein material. From the shape of the four curves in Fig. 1 destruction would seem to be negligible over long periods of time unless hydrolysis and destruction were taking place at exactly the same rate, which seems unlikely.

On the basis of these results, the conditions adopted for the method were as follows:

#### *Method*

1.25 gm. of desiccated thyroid are hydrolyzed for 18 hours with 100 cc. of 2 N NaOH in a 300 cc. short neck Kjeldahl flask, fitted with a reflux condenser and heated by a micro burner. (The use of a potassium thiocyanate bath is recommended to prevent overheating of the bottom of the flask with subsequent breakage during the long hydrolysis.)

After cooling to room temperature the hydrolysate is quantitatively transferred to a 250 cc. separatory funnel and shaken *gently* for a few minutes with an equal volume, 100 cc., of butyl alcohol which has been purified by distillation from NaOH. Violent shaking causes the formation of emulsions which require a long period of time for separation. After standing 1 hour the layers are separated, the aqueous layer being transferred to a second 250 cc. separatory funnel, and shaken a second time with 100 cc. of butyl alcohol. After standing again the layers are separated, and the first and second butyl alcohol fractions are filtered successively through

glass wool into a 500 cc. separatory funnel. The funnels are washed with 5 cc. portions of butyl alcohol. The combined butyl alcohol fractions are shaken with an equal volume, 210 cc., of N NaOH, allowed to stand, and separated. The NaOH layer is transferred to a second 500 cc. funnel and shaken with one-half its volume, 100 cc., of butyl alcohol, allowed to stand, and separated. The butyl alcohol fractions are quantitatively transferred to a 500 cc. flask and evaporated under reduced pressure to small volume. The residue is transferred quantitatively to a nickel dish and the flask washed with several small portions of butyl alcohol. 5 cc. of 50 per cent NaOH are added, the solution evaporated on a hot plate until the butyl alcohol is entirely gone, and the residue ashed over a free flame. Care should be taken in ashing to use a low flame, in no case to allow the dish to become red-hot, and to consider the ashing complete when the melt has changed from caramel to clear, all bubbling has ceased, and the bottom of the dish has become coated over with nickel oxide. Particles of carbon left in the melt need not be completely ashed as they do not interfere with the determination of iodine.

After cooling, the melt is dissolved in hot water and filtered hot through a Gooch crucible into a 125 cc. Erlenmeyer flask. After thorough washing with hot water (volume 50 to 60 cc.) 2 drops of a 1 per cent solution of sodium bisulfite are added, then 50 per cent H<sub>2</sub>SO<sub>4</sub> until acid to methyl orange with 3 drops in excess, and the solution boiled gently for a few minutes. 3 cc. of saturated bromine water are added, the solution boiled until colorless, and cooled under running water. 6 drops of 90 per cent phenol are added to remove the last traces of bromine, an excess of potassium iodide crystals is added, and the solution titrated with 0.01 N sodium thiosulfate with starch as an indicator. This strength of thiosulfate has been found to be stable if made up to contain 5 cc. of N NaOH per liter.

In a series of 52 such analyses the mean discrepancy between duplicates was 2.0 per cent, the maximum discrepancy was 5.6 per cent.

#### *Isolation of Pure Thyroxine*

As a further check on the validity of the method, we have attempted the isolation of pure thyroxine from the final butyl

alcohol fraction obtained as above from larger scale hydrolyses in which a specimen of partially purified thyroglobulin was used instead of desiccated thyroid. Butyl alcohol takes up a considerable amount of the black, tarry material formed during the alkaline hydrolysis. This tarry material must be removed before the thyroxine can be crystallized, but we were unable to remove it without serious loss of iodine. The best results were obtained in the following manner. The final butyl alcohol layer was evaporated under reduced pressure and the residue taken up in water. The hot solution was treated cautiously with barium hydroxide solution till the tar was precipitated, then quickly filtered or centrifuged. The clear, yellow, supernatant liquid was acidified just to the turning point of Congo red. The precipitate was collected and heated with 40 per cent barium hydroxide for a short time (about an hour) and thereafter the procedure of Harington (5) was followed. The yields of thyroxine obtained in this way were far from quantitative. In the best case 54 per cent of the total iodine in the butyl alcohol fraction was recovered as pure crystalline thyroxine.

A detailed account of this preparation is as follows: 100 gm. of thyroglobulin (570 mg. of  $I_2$ ) were boiled 18 hours with 2000 cc. of 2 N NaOH. Since this experiment was of a preparative nature and not intended to be a quantitative separation, the hydrolysate was for the sake of simplicity extracted only once with an equal volume of butyl alcohol, from which, on the basis of the distribution ratios, we should expect approximately 90 per cent of the thyroxine and 3 per cent of the diiodotyrosine to pass into the butyl alcohol layer. The butyl alcohol solution was washed with 500 cc. of N NaOH which should have reduced the diiodotyrosine to a negligible amount while removing only about 2 per cent of the thyroxine. The butyl alcohol was removed under reduced pressure and the residue taken up in 300 cc. of water. Analysis showed the presence of 105 mg. of iodine. The solution was heated to boiling and treated with saturated barium hydroxide solution till precipitation of the dark pigmented material seemed complete and then quickly filtered while hot. The filtrate, which contained 84 mg. of iodine, was made just acid to Congo red; whereupon all but 3 mg. of the iodine was precipitated. The precipitate was dissolved in 100 cc. of water with the help of a few drops of ammonia.

Barium hydroxide to make a 40 per cent solution was added and the solution was heated 1 hour on the steam bath and filtered. The precipitate was saved. The filtrate which contained 26 mg. of iodine was acidified to Congo red with HCl and the resulting precipitate retreated with 40 per cent  $\text{Ba}(\text{OH})_2$  in a smaller volume. The filtrate from the second crop of barium-insoluble material contained only 8 mg. of iodine and was discarded. The two crops of barium-insoluble material were combined and treated with NaOH and  $\text{Na}_2\text{SO}_4$ , according to Harington (5). On acidification of the alkaline filtrate from the barium sulfate, crude thyroxine separated in partly crystalline form. This was purified by dissolving in hot 0.5 per cent sodium carbonate solution and crystallizing out the sodium salt, and finally by crystallizing the free thyroxine from alcohol on acidification with acetic acid. The total yield was 89 mg. of thyroxine which analyzed 98 per cent pure, thereby accounting for 54 per cent of the total iodine of the butyl alcohol fraction as crystallized thyroxine.

#### *Comparison with Method of Harington and Randall*

The method of Harington and Randall is based on the assumption that all of the thyroxine and none of the diiodotyrosine is present in the acid-insoluble fraction after partial hydrolysis by alkali. We cannot agree with this. If the acid-insoluble precipitate is collected and further hydrolyzed by boiling 18 hours with 2 N NaOH and the resulting solution fractionated with butyl alcohol, it is found that about half the iodine remains in the aqueous layer; *i.e.*, does not behave as does thyroxine. This fraction of the iodine is in organic combination, is not precipitated by acidification, and its solution gives a strong nitrous acid reaction; hence we conclude that it represents diiodotyrosine. A description of such an experiment follows.

100 gm. of desiccated thyroid were boiled 4 hours with 1 liter of 2 N NaOH, cooled, and acidified to pH 5 with HCl, allowed to stand 6 hours, and filtered. The precipitate was dissolved in 110 cc. of 2 N NaOH. Analysis of 2 cc. aliquots of this solution showed the presence of 142 mg. of iodine in the acid-insoluble fraction. The solution was boiled 18 hours, and then extracted with butyl alcohol in the manner described. The combined butyl alcohol solutions were evaporated and taken up in 106 cc. of water. This solution

was found to contain only 70.6 mg. of iodine. Approximately half of Harington and Randall's acid-insoluble iodine was in the form of thyroxine. The aqueous alkaline layer (volume 100 cc.) after the treatment with butyl alcohol, was acidified to Congo red with  $H_2SO_4$ . No precipitate formed, but the solution gave a strong nitrous acid test. Analysis for inorganic iodide by the method previously described (6) showed the presence of only 5 mg. of iodine in this form; the rest, 66 mg., we conclude must have been present as diiodotyrosine.

The figures published by Harington and Randall (2) for the thyroxine content of eight samples of commercial thyroid preparation indicate a much higher ratio of thyroxine iodine to total iodine than we found when we applied our method to a series of 52 human thyroids, the data of which are given in Table VI. When the same sample of thyroid was analyzed by both methods, the results were also quite different. A sample of commercial thyroid gave by the method of Harington and Randall 45.4 per cent; by our method 23.3 per cent of the total iodine as thyroxine iodine. Another sample showed 48 per cent by the procedure of Harington and Randall and 23 per cent by ours.

#### *Thyroxine Content of Normal Human Thyroids*

The method was applied to the examination of a series of adult human thyroids, mainly from traumatic cases with sudden death. Histological examination of the glands was made by Dr. A. B. Gutman of the Department of Medicine of this school, to whom we express our sincere thanks. Pieces of the glands removed for histological study (about 0.5 gm. of fresh tissue) are not included in the weights recorded in Table VI.

The glands were kept on ice from autopsy until collection could be made, an interval of from 1 to 3 days. They were then minced finely, dried at 78-80° in an electric oven for 18 hours, and ground to a uniformly fine consistency. They were defatted by extraction with petroleum ether in a Soxhlet apparatus for 9 hours. In a test sample analyses were made of the petroleum ether extract and of the desiccated thyroid being extracted. The loss of iodine by extraction was negligible.

The glands after defatting were brought to constant weight at 60° in the electric oven and then analyzed according to the method

described above for thyroxine, 1.25 gm. samples being used except in the case of the smallest glands where 1 gm. samples were used. 0.25 gm. samples were used for the determination of total iodine.

Histological examination of the glands showed varying degrees of autolysis, due, no doubt, to the unavoidable delay in the handling of the glands. To determine whether the total iodine or the thyroxine content was affected by autolysis, three of the larger glands were divided into two parts after mincing. One-half was dried immediately while the second half was kept 1 week in the ice box (a length of time double the delay to which our glands were subjected) and then dried. Each half was defatted, brought to constant weight, and analyzed in the usual manner. The results are shown in Table V.

TABLE V  
*Effect of Autolysis of Gland on Total Iodine and Thyroxine Iodine*

Gland No.	Total I <sub>2</sub> , per cent		Thyroxine I <sub>2</sub> , per cent of total	
	Before	After	Before	After
44	0.106	0.107	20.5	21.5
52	0.348	0.351	29.1	29.5
54	0.212	0.207	26.4	27.0

The close agreement between the pairs allows us to conclude that neither the total iodine nor the thyroxine content is affected by autolysis.

The detailed results of the analyses are shown in Table VI. We have not attempted to correct the results of thyroxine determinations for the presumable 15 per cent destruction of thyroxine during hydrolysis of the gland.

In the series of 52 human thyroids the mean content of total iodine was 0.174 per cent with a mean deviation of 0.066; whereas the percentage of the total iodine which was in the form of thyroxine showed less scatter, the mean being 25.2 per cent with a mean deviation of 4.9.

#### SUMMARY

1. Thyroxine may be separated from the other iodine compounds which are present in an alkaline hydrolysate of the thyroid gland by extraction with butyl alcohol.

## Thyroxine

TABLE VI  
*Total Iodine and Thyroxine Iodine in Human Thyroids*

Case No.	Sex	Age	Cause of death	Weight of gland		Total I <sub>2</sub> per gm. dry weight	Thyroxine I <sub>2</sub> as per cent of total I <sub>2</sub>	Thyroxine I <sub>2</sub> in whole gland	<i>Histology</i> (Marie's terminology)*
				Wet	Dry				
1	M.	60	Trauma	15	3.33	1.81	0.466	25.7	Normal; early involution
2	"	45	Unknown poison	20	4.71	0.97	0.205	21.1	" " "
3	F.	50	Trauma	26	5.69	1.74	0.464	26.7	Adenoma; involution
4	"	50	"	17	3.76	2.75	0.828	30.2	Normal; early involution
5	M.	60	"	40	9.05	1.13	0.215	19.0	" " "
6	"	50	"	20	2.86	1.00	0.132	13.2	Adenoma; early hyperplasia
7	"	49	"	40	8.48	1.22	0.248	20.3	Normal; early involution
8	"	45	Lobar pneumonia	20	3.63	1.54	0.349	34.8	"
9	"	58	Trauma	9	1.99	1.94	0.630	32.5	1.25 "
10	"	20	Not obvious	20	4.23	1.69	0.500	29.6	2.11 "
11	"	30	Trauma	20	5.66	2.37	0.742	31.3	4.15 "
12	"	25	Acute alcoholism	15	3.45	1.83	0.565	30.9	1.95 "
13	"	50	Trauma	25	5.28	2.36	0.637	27.0	3.37 "
14	"	30	"	13	2.64	0.64	0.074	11.6	0.20 "
15	"	50	"	18	4.19	2.05	0.368	18.0	1.54 "
16	"	50	Alcoholism	16	2.84	1.31	0.408	31.2	1.16 "
17	"	45	"	10	1.58	1.18	0.202	17.1	0.32 Early involution
18	"	45	"	17	2.89	1.44	0.233	16.2	0.67 Beginning hyperplasia
20	F.	35	Trauma	17	2.70	1.68	0.454	27.0	1.22 Poor specimen
21	M.	28	"	18	3.48	1.26	0.334	26.5	1.16 Some areas hyperplastic
22	"	25	Acute suppurative meningitis	23	5.12	2.03	0.592	29.2	3.03 Normal; resting

23	M.	52	Alcoholism	15	3.12	4.21	1.19	28.3	3.72	Early involution; basophilic colloid
24	"	28	Trauma	18	3.53	1.63	0.463	28.4	1.64	Normal; resting
25	F.	35	Gas poisoning	21	4.89	1.44	0.365	25.3	1.79	Adenoma; involution
26	M.	29	Trauma	22	6.00	2.33	0.623	26.8	3.74	Normal
27	"	40	"	30	6.25	1.21	0.272	22.5	1.70	"
28	"		Alcoholism	15	2.88	0.80	0.134	16.7	0.39	Considerable hyperplasia with papillary projections
29	"	24	Trauma	16	3.46	1.56	0.385	24.7	1.33	Normal
30	"	50	"	10	2.32	1.24	0.284	22.9	0.66	Several areas early hyperplasia
31	"	50	"	20	3.54	1.53	0.378	24.7	1.34	Early involution
32	F.	21	Not ascertained	24	6.82	2.16	0.580	26.9	3.95	Normal; resting
33	M.	47	Trauma	28	6.32	2.94	0.766	26.1	4.84	"
34	"	42	Alcoholism	37	8.62	1.38	0.337	24.5	2.91	1 adenoma; early involution
35	"	21	Drowning	15	3.82	1.80	0.544	30.3	2.08	Normal; resting
36	"		Trauma	37	8.90	1.57	0.431	27.5	3.84	"
37	"	50	Coronary embolism	18	3.50	1.61	0.461	28.6	1.61	Early involution
38	"	55	Trauma	12	2.80	1.68	0.407	24.3	1.14	Normal; 1 area of beginning hyperplasia
39	"	55	Drowning	20	3.61	1.31	0.322	24.6	1.16	Early hyperplasia; many papillary projections
40	"	32	Trauma	19	4.50	1.43	0.352	24.6	1.58	Normal
41	F.	40	"	12	2.60	2.04	0.576	28.2	1.50	"
42	M.	35	Alcoholism	22	4.92	1.46	0.400	27.4	1.97	" resting
43	"	45	Trauma	16	3.43	1.43	0.335	23.5	1.15	Early hyperplasia; many papillary projections
44	"	40	"	17	3.46	1.06	0.217	20.5	0.75	Hyperplasia marked in several areas
45	"	45	Alcoholism	9	2.18	2.64	0.808	30.6	1.76	Normal; beginning hyperplasia

## Thyroxine

TABLE VI—Concluded

Case No.	Sex	Age yrs.	Cause of death	Weight of gland		Total I <sub>s</sub> per gm. dry weight	Thyroxine I <sub>s</sub> per cent of total I <sub>s</sub>	Thyroxine I <sub>s</sub> in whole gland	Histology (Marine's terminology)*
				Wet gm.	Dry gm.				
46	M.	50	Lobar pneumonia	22	4.65	2.53	0.641	25.4	Normal; resting
47	"	19	Trauma	31	8.33	1.45	0.349	24.1	"
49	"		Alcoholism	17	2.89	0.33	0.06	18.3	Pronounced hyperplasia; hyperemia
50	"	48	Pneumonia	19	4.23	1.23	0.328	26.6	Early involution
51	F.	26	Trauma	17	4.39	2.53	0.744	29.4	Normal; resting
52	"	19	"	22	5.75	3.48	1.02	29.1	Occasional papillary projections; beginning hyperplasia
53	M.	50	Drowning	47	10.73	2.33	0.553	23.7	Normal; resting
54	"	36	Trauma	29	6.87	2.12	0.559	26.4	"

\* The glands were designated as normal unless variations were pronounced. Such variations are noted.

2. On the basis of this finding, a method for the determination of thyroxine in desiccated thyroid gland is described.

3. There is unavoidable destruction of some of the thyroxine during the alkaline hydrolysis. The amount of this destruction as judged by recovery experiments is not more than 15 per cent of the total amount of thyroxine.

4. In a series of 52 human thyroids the mean thyroxine content (as iodine) was 25.2 per cent of the total iodine (without correction for the presumable 15 per cent destruction of thyroxine during hydrolysis).

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# THE MECHANISM OF THE PRODUCTION OF THIOL ACIDS (R—S—H) AND SULFONIC ACIDS (R—SO<sub>3</sub>—H) FROM DITHIO ACIDS (R—S—S—R)

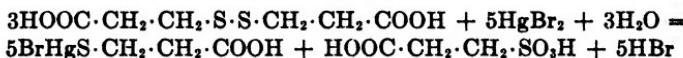
## II. THE ACTION OF MERCURIC SALTS\*

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(Received for publication, May 29, 1931)

The investigation of the mechanism of the oxidation and reduction reaction in which dithio acids (R—S—S—R) are converted simultaneously into thiol acids (R—S—H) and sulfonic acids (R—SO<sub>3</sub>—H) has been extended to include a study of the reaction between dithio acids and mercuric salts. The results of a quantitative analytical procedure in which the principal products of the reaction were isolated and identified indicate that the reaction between dithiodihydracrylic acid and mercuric bromide proceeds to at least 75 per cent according to the following equation.



This reaction of dithio acids and their reactions with other molecules or metallic ions (2, 11, 12, 16) indicate that the transformation may be represented by



in which the formation of R—S—H and R—SO<sub>3</sub>—H is accelerated by the removal of the R—S—H present as an insoluble derivative

\* Presented before the American Society of Biological Chemists at Montreal, April, 1931.

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of  $\text{Ag}^+$ ,  $\text{Hg}^{++}$ , or  $\text{Cu}^+$ ; as a slightly ionized derivative of  $\text{BrHg}^+$ ; or by reoxidation to  $\text{R}-\text{S}-\text{S}-\text{R}$  as by  $\text{Cu}^{++}$  or  $\text{I}_2$ .

#### EXPERIMENTAL

The results of the preliminary experiments with cystine, dithiodiglycolic acid, dithiodilactic acid, and dithiodihydracrylic acid reacting with mercuric salts may be summarized as follows: The amounts of thiol derivatives formed, as estimated by  $\text{KIO}_3$  titration of the solution of the precipitate in  $\text{KI}$  and  $\text{HCl}$ , vary greatly with the experimental conditions. The relatively higher concentrations, higher temperatures, and lower acidities, while increasing the rate of the reduction to thiol, tend to increase to a greater extent the side reactions. Some reduction to  $\text{Hg}^+$  and  $\text{Hg}$  usually results, as evidenced by the formation of  $\text{Hg}$  when the precipitate is treated with  $\text{HI}$ ; less reduction of  $\text{Hg}^{++}$  occurs with the halides than with other mercuric salts. The order of addition of the reagents is important; greater reduction of  $\text{Hg}^{++}$  occurs if the mercuric solution is added to the dithio acid than when the addition is reversed.

#### *Dithiodihydracrylic Acid Reaction with Mercuric Bromide*

By properly adjusting the conditions for the reaction between  $\text{HgBr}_2$  and dithiodihydracrylic acid, a fraction consisting of a small amount of amorphous reduced material and another consisting of clear square plates of the thiol derivative could be isolated.

100 cc. of 0.1 N HBr, 3.6 gm. of  $\text{HgBr}_2$ , and 1850 cc. of water were heated in a covered 3 liter beaker in a steam bath to 90–95° and 50.00 cc. of 0.0400 N disodium dithiodihydracrylate (11) were added. The heating was for 36 hours (Experiments 7 through 9) or for 72 hours (Experiments 1 through 6), water being added occasionally to replace that which had evaporated. The mixture remained clear for the first few minutes then an opalescence occurred and in about 10 minutes the mixture was relatively opaque due to a fine white precipitate which turned gray and after several hours settled out. This material (0.025 to 0.040 gm.) was rapidly filtered off on a preheated suction filter. It appears to be a product of the reaction between the  $\text{HgBr}_2$  and the dithio acid or of some reaction occurring with it, rather than a result of an interaction of the  $\text{HgBr}_2$  with the formed thiol deriva-

tive, since no precipitate was formed in the separate reaction between the thiol acid and  $HgBr_2$ . When treated with  $NH_4OH$  it gave the blackening characteristic of mercurous salts; it was probably a mixture of several reduced substances. There was no essential difference in the quantity of thiol derivative isolated when the time was doubled, so the reaction may be assumed complete and the action of atmospheric oxygen negligible.

*Isolation of Thiol Derivative*—The filtrate from the gray material was allowed to cool to  $10^\circ$ , whereupon clear square plates of the  $BrHgS \cdot R$  derivative crystallized out. The total amount formed was estimated as the sum of that isolated on a sintered glass crucible, washed with acetone (8), and weighed (80 to 90 per cent); that remaining in the beaker and determined by titration (8 to 18 per cent); and that in the filtrate (2 to 4 per cent) which was estimated from the solubility (0.010 to 0.015 gm. per liter).

*Analysis of Thiol Derivative. Thiol Determination*—0.1 to 0.2 gm. of material was dissolved in excess KI and HCl and titrated (11) with 0.1000 N  $KIO_3$ . Found, —SH (average of twelve experiments), 8.57 per cent; theoretical, 8.58 per cent.

*Mercury Determination*—A 0.2 gm. sample was dissolved in  $Na_2S$  and  $NaOH$  and the  $HgS$  precipitated by boiling with excess  $NH_4NO_3$  until most of the  $NH_3$  had been removed (14). Weighed  $HgS$ . Found, Hg (average of six experiments), 52.0 per cent; theoretical, 52.02 per cent.

*Bromine Determination*—A 0.2 to 0.3 gm. sample in 20 cc. of water was treated with  $Na_2O_2$ . The excess was decomposed by boiling and  $SO_2$  was added until acid to litmus. Excess  $SO_2$  was expelled,  $HNO_3$  added, and  $AgBr$  precipitated by  $AgNO_3$  (14). Weighed  $AgBr$ . Found, Br (average of six experiments), 20.6 per cent; theoretical, 20.73 per cent.

*Isolation of Sulfonic Acid*—Since the sulfonic acid is isolated as a barium salt from alcoholic solution (11, 13), it is necessary to remove from the filtrate from the  $BrHgS \cdot R$  those ions which might form contaminating precipitates.

To remove  $Hg^{++}$ ,  $H_2S$  was passed into the solution, the mixture was evaporated to about 500 cc., and filtered. Then  $Br^-$  was removed as  $TlBr$  by adding 5.2 gm. of  $Tl_2SO_4$ , heating to coagulate, then cooling and filtering. Thallous rather than silver salt (11) was used because it did not react significantly with the dithio

acid under these conditions. The  $\text{SO}_4^-$  and excess  $\text{Tl}^+$  were precipitated by 7 gm. of  $\text{BaI}_2$  as  $\text{BaSO}_4$  and  $\text{TlI}$  and removed by filtration. After evaporation to 50 cc., centrifugalization, and then evaporation to 10 cc., 100 cc. of ethyl alcohol were added.

TABLE I  
*Analytical Data for Isolation and Identification of Products of the Reactions*

Experiment No.	Substances isolated		Analysis of $\text{BrHgS}\cdot\text{R}$		
	Total $\text{BrHgS}\cdot\text{R}$	$\text{R}-\text{SO}_3-\text{H}$ (Ba salt)	Br	Hg	-SH
Dithiodihydracrylic acid reacting with mercuric bromide					
1	0.4828	0.0948	per cent	per cent	per cent
2	0.4819	0.0938		52.53	8.57
3	0.4774	0.0939		51.63	8.55
4	0.4878	0.0985		52.66	8.53
5	0.4773	0.0884		51.15	8.59
6	0.4801	0.0874		51.73	8.53
7	0.4779		20.51	52.20	8.50
8	0.4861		20.61		8.56
9	0.4931		20.58		8.50
10			20.17		8.69
11			20.62		8.72
12			20.81		8.54
Average.....	0.483	0.093	20.6	52.0	8.53
Theoretical....	0.641	0.096	20.73	52.02	8.58
Thiohydrazacrylic acid reacting with mercuric bromide					
21	1.4947		20.62	51.92	8.56
22	1.4647		20.68	51.69	8.57
23	1.4660		20.30	52.00	8.60
24	1.4984		20.57	52.01	8.59
25	1.4472		20.89	52.41	8.62
Average.....	1.480		20.6	52.0	8.59
Theoretical....	1.505		20.73	52.02	8.58

The material formed was reprecipitated by alcohol from aqueous solution containing  $\text{BaI}_2$  and  $\text{HI}$ , which prevents contamination with thiol or dithio salts, until no further significant decrease in weight in the final anhydrous salt resulted. Anhydrous salt,

dried at 180°, Ba( $\text{—OOC}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{SO}_3\text{—}$ ), isolated (average of six experiments), 0.093 gm.; theoretical, 0.096 gm.

The results of the isolations and analyses have been compared (see Table I) to the theoretical values based on the equation previously cited; and while other reduction reactions, such as those accompanying the reduction of  $\text{Hg}^{++}$ , may have contributed a very small share of the final products, the calculations indicate that this is the principal reaction.

*Analysis of Barium Sulfonate*—The material from the six experiments was combined, dissolved in a minimum amount of hot water, centrifugalized, and allowed to cool slowly, crystallizing out the  $5\text{H}_2\text{O}$  salt (11, 13). This was filtered off on a sintered glass crucible and dried under ordinary atmospheric conditions; there was recovered as hydrated salt 0.2972 gm. To the filtrate was added 10 times its volume of alcohol and the precipitate was filtered off and heated at 180°; obtained, 0.3020 gm. Total recovery, calculated as anhydrous salt, 0.5287 gm.; theoretical, 0.5568 gm.; per cent of recovery, 95.

Micro analyses of the  $5\text{H}_2\text{O}$  salt gave these results: 9.285 mg. of substance gave 3.134 mg. of  $\text{CO}_2$  and 3.075 mg. of water, leaving a residue of 5.620 mg. 8.076 mg. of substance gave 4.980 mg. of  $\text{BaSO}_4$  by precipitation with  $\text{H}_2\text{SO}_4$ . 7.910 mg. of substance lost 1.839 mg. on heating at 180°.

Found (per cent). C 9.20, H 3.70, ash 60.52, Ba 36.29, moisture 23.24  
Theoretical. C 9.49, H 3.71, ash ( $\text{BaSO}_4$ ) 61.50, Ba 36.20, ( $5\text{H}_2\text{O}$ ) 23.73

#### *Thiohydracrylic Acid Reaction with Mercuric Bromide*

The acid was standardized (11) by titration with NaOH for acid value and  $\text{KIO}_3\text{-KI}$  for reducing value. The ratio of the thiol titer to the acid titer was 1.03 (average of four determinations). Shinohara (15) states that cystine can be oxidized to cysteic acid by iodine and a similar reaction may be occurring to a slight extent here (12).

1875 cc. of water, 100 cc. of 0.1 N HBr, and 3.60 gm. of  $\text{HgBr}_2$ , were heated to 95° and 25.00 cc. of 0.1561 N thiohydracrylic acid were added. On cooling to 10°, clear square plates formed and their quantity was estimated as described. No gray or white amorphous material, such as was formed in the reaction with the dithio acid, appeared after heating for several hours.  $\text{BrHgS}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$  isolated, 1.480 gm. (average of five

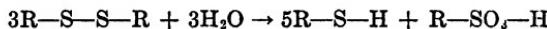
preparations); theoretical, 1.505 gm.; per cent of theoretical, 98.3.

The results of the analyses (see Table I) and the physical properties of the compound prepared from thiohydrylic acid indicate that it is identical with the material isolated after the reaction between dithiodihydracrylic acid and  $HgBr_2$  and give further evidence that the material from the dithio acid is a thiol derivative. The equation for the reaction is



#### DISCUSSION

Vickery and Leavenworth (16) showed that on treating cystine in  $H_2SO_4$  with  $Ag_2SO_4$ , 70 to 85 per cent of the original cystine ( $R-S-S-R$ ) could be recovered as a silver derivative of cysteine ( $R-S-H$ ) and 1.63 per cent as a cysteic acid-copper salt ( $R-SO_3-H$ ). For that part of the reaction yielding cysteic acid, they postulated the equation

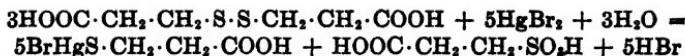


Calculated on the basis of the cysteic acid found, this reaction was proceeding to an extent of only 10 per cent.

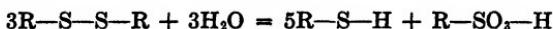
Preisler and Preisler (11) established definitely the validity of the equation by showing that, when  $Ag_2SO_4$  reacts with  $HOOC \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COOH$ , characteristic derivatives of  $HOOC \cdot CH_2 \cdot CH_2 \cdot SH$  and  $HOOC \cdot CH_2 \cdot CH_2 \cdot SO_3H$  are obtained in yields amounting to 99 to 100 per cent in accordance with the equation.

Andrews and Wyman (2) prepared, by the interaction of cystine in  $H_2SO_4$  with  $HgSO_4$ , a mercury derivative which on analysis showed that the  $Hg:N$  ratio was close to 3:2, but to which no satisfactory formula could be assigned. This substance when treated with  $H_2S$  gave cysteine and no free S could be detected in the  $HgS$  formed, which indicated that the reduction of the cystine had probably occurred before the  $H_2S$  reaction rather than by it; a mercury derivative of cysteine apparently results from the reaction between  $HgSO_4$  and cystine.

The present work has established that mercuric halides react with dithio acids, producing thiol acids and sulfonic acids. The reaction between dithiodihydracrylic acid and  $HgBr_2$  occurs to at least 75 per cent according to the equation



The investigations of the effects of certain molecules or ions on dithio acids indicate that the transformation is accelerated by substances which decrease the amount of R—S—H which appears to form when water reacts with the dithio compounds according to the equation



The formation of highly insoluble compounds of R—S—H by reaction of the metallic ions, Ag<sup>+</sup> or Hg<sup>++</sup>, or very slightly ionized compounds by reaction with mercuric halide ions, BrHg<sup>+</sup>, causes the reaction to proceed to R—S—H and R—SO<sub>3</sub>—H formation. The reaction of R—S—S—R with Cu<sup>++</sup> yields a dicuprous derivative, CuS·CH<sub>2</sub>·CH<sub>2</sub>·COOCu and R—SO<sub>3</sub>—H; either the insolubility of the dicuprous salt or the reoxidation of R—S—H to R—S—S—R by the Cu<sup>++</sup> would cause the reaction to proceed with the formation of sulfonic acid (12). Iodine, which Shinohara (15) finds converts cystine into cysteic acid and which also oxidizes dithiodihydracrylic acid to β-sulfopropionic acid (12), may be considered to be reacting through a similar mechanism by reformation of R—S—S—R by oxidation of the R—S—H formed. In view of the newer experimental findings, the hypothesis of intermediary silver peroxide formation previously proposed (11) may be discarded.

This reaction is suggested as a possible mechanism in the formation of sulfonic acids in the organism, since the organism need only be able to reoxidize R—S—H to R—S—S—R, a relatively easy oxidation to perform, to be capable of sulfonic acid formation, which usually requires powerful oxidants. Of interest also is the possible diversion of the oxidative phase (R—SO<sub>3</sub>—H formation) to the oxidation of, or the reductive phase (R—S—H formation) to the reduction of other substances than the sulfur compounds themselves and its possible assistance in modified form, in the intermediary metabolism of some of the many materials utilized by the organism.

Because of the close relationship between thiohydracrylic acid and cysteine, the information obtained regarding the mercuric halide derivative of thiohydracrylic acid may be of assistance in

establishing the constitution of the cysteine-mercuric chloride compound obtained by Neuberg and Mayer (9) to which they assigned the formula  $[\text{CH}_2 \cdot \text{SHgCl} - \text{CH} \cdot \text{NH}_2(\text{HCl}) - \text{COOH}]_2 \cdot \text{HgCl}_2$ . The combination might better be represented as the amine salt,  $(\text{CHgS} \cdot \text{CH}_2 \cdot \text{CHNH}_3^+ \cdot \text{COOH})_2\text{HgCl}_4^-$ , the di-cysteine salt of tetrachloromercuric (7) acid,  $\text{H}_2\text{HgCl}_4$ , since thiohydracrylic acid has been demonstrated to form no  $\text{HgCl}_2$  type of addition compound and the  $\text{ClHg}^+$  and  $\text{HgCl}_4^-$  ions form the largest (6) proportion of the ionized  $\text{HgCl}_2$  used for the precipitation.

The failure to obtain a quantitative recovery of certain  $\text{R}-\text{S}-\text{H}$  or  $\text{R}-\text{S}-\text{S}-\text{R}$  compounds after treating with metallic salts may possibly be explained by the occurrence of the sulfonic acid-forming reaction. Cystine cannot be quantitatively recovered after precipitation by mercuric salts (1, 3, 9, 10) or by silver salts (16) which is no doubt due to sulfonic acid formation. Glutathione isolation and purification depends (4, 5) upon the formation of certain copper- or mercury-containing derivatives, so that if some glutathione exists originally as the  $\text{R}-\text{S}-\text{S}-\text{R}$  form or if this form results from oxidation of  $\text{R}-\text{S}-\text{H}$  occurring during the extractions, loss might occur by the formation of  $\text{R}-\text{SO}_3-\text{H}$  from the  $\text{R}-\text{S}-\text{S}-\text{R}$ .

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# ON THE CHEMISTRY OF THE CONJUGATION OF BENZOIC ACID\*

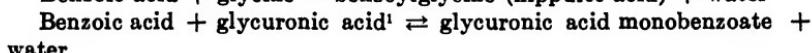
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(Received for publication, November 16, 1931)

In trying to find a reason why the dog excretes three-quarters of the ingested benzoic acid as glycuronic acid monobenzoate and only one-quarter as hippuric acid, the most satisfactory explanation seems to be found in the view that the processes of conjugation are chemical reactions, catalyzed by enzymes, and obeying the law of mass action. It should therefore be possible to study these mechanisms as one does any reversible reaction; it should be borne in mind, however, that these chemical processes are occurring in a complex organism and are subjected to forces which are poorly understood. It is not to be expected that one can obtain data which may be interpreted with mathematical precision, but the experimental results should at least qualitatively correspond to the theoretically expected findings. The two reactions that express the chemistry of the conjugation of benzoic acid are



On inspecting these equations, it is seen that the two reactions are mutually dependent upon each other, since benzoic acid is

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

<sup>1</sup> There is no experimental evidence that glycuronic acid is first formed and then conjugated. It seems more reasonable to consider glycuronic acid as the end-product derived from a carbohydrate precursor. The term, glycuronic acid, as used subsequently in this paper is to be considered synonymous with this unknown precursor.

common to both, and furthermore that the partitioning of benzoic acid between these two types of conjugation must depend upon three basic factors: (1) the rate of the elimination of the end-products, hippuric acid and glycuronic acid monobenzoate; (2) the concentration and supply of glycine and of glycuronic acid; (3) the speeds of reaction between benzoic acid and glycine, and between benzoic acid and glycuronic acid.

Unless the products of a reversible reaction be promptly removed, they will tend to reach a concentration at which the speed of the reverse reaction will equal the speed of the direct reaction, and a state of equilibrium will be established. Should, therefore, hippuric acid and glycuronic acid monobenzoate be retained in

TABLE I  
*Formation of Hippuric Acid Following Intravenous Administration of  
 Glycuronic Acid Monobenzoate*  
 Dog C, weight 7 kilos.

Period hrs.	Benzoic acid excreted as*	
	Hippuric acid gm.	Glycuronic acid monobenzoate gm.
6	0.16†	0.57
12	0.09	0.12

\* 2.5 gm. of glycuronic acid monobenzoate, equivalent to 1.0 gm. of benzoic acid, were given intravenously.

† Hippuric acid was definitely identified. The blank for a 12 hour fasting period was found to be 0.04 gm. of benzoic acid.

sufficient amounts to bring about the reverse reaction, the difference in the equilibrium constants of the two reactions would become an important determinant of the ultimate ratio of benzoic acid combined with glycine to that combined with glycuronic acid. The elimination of both glycuronic acid monobenzoate and hippuric acid is, however, in the normal dog so prompt that it can almost be taken for granted that the rate of excretion nearly approximates the rate of synthesis. Nevertheless, it can be demonstrated experimentally that both reactions are reversible. When glycuronic acid monobenzoate is injected intravenously into a dog, a definite quantity of hippuric acid will be excreted (Table I); similarly, when hippuric acid is injected a small fraction will be

excreted as glycuronic acid monobenzoate as indicated by the reducing power of the urine (Table II). From this it can be seen that if the kidney threshold for glycuronic acid monobenzoate be high, as it probably is in some species, the concentration of this compound would tend to become high enough to bring about a considerable reversal of the conjugating process and so decrease the production of glycuronic acid monobenzoate.

The amount of preformed glycine in the body is unquestionably low and easily exhausted. Furthermore, the capacity of the dog to synthesize this amino acid is distinctly limited. Nevertheless, the maximum quantity of glycine that the animal can produce is

TABLE II  
*Formation of Glycuronic Acid Monobenzoate Following Intravenous Administration of Hippuric Acid*

Dog 5, weight 10 kilos.

Period	Benzoic acid excreted as*	
	Hippuric acid	Glycuronic acid monobenzoate
hrs.	gm.	gm.
2	2.38	0.11
4	0.75	0.27†
6	0.45	0.11
8	0.05	0.11

\* 7.35 gm. of hippuric acid, equivalent to 5.0 gm. of benzoic acid were given intravenously.

† Urine definitely reduced Benedict's solution.

definitely higher than the amount excreted as hippuric acid. Thus, the excretion of glycine as hippuric acid for a 6 hour period is about 18 to 28 mg., whereas the output as phenylaceturic acid under identical conditions is about 28 to 36 mg. Therefore, the dog's power to synthesize glycine, though relatively small, does not seem to be the major factor which limits the production of hippuric acid.

It is hypothetical to speak of the concentration of glycuronic acid in the body since the uncombined acid has never been found in any organism. Little, moreover, is known either of its origin or its metabolism. The writer (1) has shown that it can be and undoubtedly is derived from carbohydrates, but that the direct con-

TABLE III  
*Various Factors Influencing Rate of Conjugation of Benzoic Acid\**

Date	Dog No.	Excretion of benzoic acid as						Remarks	
		Glycuronic acid monobenzoate			Hippuric acid				
		6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.		
1930-31		gm.	gm.	gm.	gm.	gm.	gm.		
Dec. 26	6 (10 kg.)	1.19	1.07	0.84	0.37	0.36	0.49	5 gm. benzoic acid	
Jan. 5		1.39	1.40	0.82	0.49	0.37	0.46	5 " " "	
" 10		1.19	1.18	0.83	0.76	0.68	0.55	5 " " " and 15 gm. gelatin	
" 24		1.29	1.03	0.54	0.23	0.28	0.25	5 gm. benzoic acid with protein-free diet	
Feb. 6		0.91			0.59	0.52		2.5 gm. benzoic acid	
" 7		1.13	0.54	0.21	0.60	0.57	0.04	2.5 " " "	
" 10		1.45	1.83	1.16	0.34	0.39	0.81	10 " " "	
" 17		1.01	1.05	0.46	0.39	0.81	0.54	5 gm. benzoic acid; 10 gm. gelatin 6 hrs. later	
" 21		0.66	0.98	0.63	0.34	0.38	0.85	5 gm. benzoic acid and 5 gm. borneol	
Apr. 27		1.00	1.18	0.65	0.47	0.47	0.53	5 gm. benzoic acid	
May 5		0.62	1.01	0.55	0.44	0.43	0.49	5 " " " and 5 gm. menthol	
" 7		1.15	0.75	0.63	0.40	0.55	0.48	5 gm. benzoic acid and 5 gm. menthol	
" 11		1.64	1.22	0.97	0.30	0.34		5 gm. benzoic acid	
" 7	C (7 kg.)	0.12	0.12	0.11	0.06	0.06	0.09	Routine diet	
" 8		0.96	0.95	0.60	0.26	0.31	0.38	3.5 gm. benzoic acid	
Oct. 13	2 (10 kg.)	0.50	0.59	0.96	0.18	0.24	0.38	3.5 " " " and 5 gm. menthol	
" 22		2.02			0.20			5 " " " intravenously	
Nov. 5		1.84	1.24		0.48	0.56		5 gm. benzoic acid and 5 gm. glycine intravenously	

\* Benzoic acid was fed in the form of its sodium salt with a diet consisting of casein, lard, sucrose, and bone ash. The analytical methods employed were the same as in the earlier studies (2).

version of glucose to glycuronic acid is rather unlikely. Whatever the precursors, the potential supply is relatively large. Yet, if borneol or menthol be fed simultaneously with benzoic acid, the production of glycuronic acid monobenzoate may be appreciably diminished, as shown in Table III. Both borneol and menthol are also combined with glycuronic acid and thus will deplete the available supply.

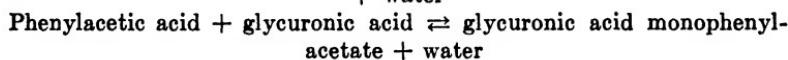
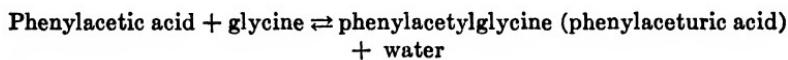
The relative speeds of the reactions between benzoic acid and glycine and between benzoic acid and glycuronic acid are probably an important factor in determining the quantitative ratios of the two types of conjugation. One must not conclude, however, *a priori* that the rate of the synthesis of glycuronic acid monobenzoate is much the faster of the two conjugating reactions because a large fraction of the benzoic acid is excreted in that form. This conclusion would logically follow if both reactions occurred simultaneously and if adequate supplies of glycine and glycuronic acid were available. It is fairly certain that the two conjugations take place in different organs and at different time intervals. The union of benzoic acid with glycuronic acid presumably takes place in the liver and perhaps also in other tissues, whereas the coupling with glycine occurs primarily in the kidney.

It appears that nearly all of the benzoic acid is promptly conjugated with glycuronic acid before the drug is again released into the general circulation, and probably very little of the acid reaches the kidney in the uncombined state. Glycuronic acid monobenzoate although rapidly excreted will undergo partial hydrolysis. The benzoic acid thus liberated is combined in the kidney with glycine, whereas the glycuronic fraction disappears and is presumably burned. It is probable that little benzoic acid combines directly with glycine, for if it did the administration of excess glycine should exert a much more pronounced effect such as occurs in the case of phenylacetic acid.

In considering the conjugation of benzoic acid in man, it should be obvious from the foregoing discussion that the process must be intrinsically the same as in the dog and that therefore the same factors must be considered. A complete answer as to why man excretes benzoic acid mainly as hippuric acid cannot be given since the various factors have not been studied as thoroughly as in the dog. The kidney threshold for glycuronic acid monoben-

zoate is probably high, for in a previous study (3) the writer found, on ingesting this compound, that only hippuric acid appeared in the urine. Little is known of the available and maximum supply of glycuronic acid. A few experiments on the output of borneol glycuronic acid indicate that the body would have more than enough to conjugate all of the 5 gm. of benzoic acid used in the author's standard experiments. The supply of glycine is comparatively large and the elimination of hippuric acid very prompt. There seems to be a marked difference in the speed of the two conjugating reactions. The union of glycine with benzoic acid appears to occur directly and rapidly. This raises the question whether the synthesis of hippuric acid may not occur in the liver and perhaps other organs beside the kidney. Further work is required before the chemical changes that benzoic acid induces in the human organism are fully understood.

The conjugation of phenylacetic acid in the dog differs markedly from that of benzoic acid. It is excreted combined largely with glycine and only to a limited extent with glycuronic acid. As in the case of benzoic acid, this dual conjugation must be interpreted from the standpoint of the law of mass action. The two basic equations are



Again the three determinants, *i.e.* the rate of the elimination of the end-products, the concentration of the conjugating components, and the speeds of the reaction, must be considered. Phenylaceturic acid is of course rapidly eliminated, but the kidney threshold for glycuronic acid monophenylacetate appears to be higher than for the corresponding benzoic acid compound. The available supply of glycine and glycuronic acid is obviously the same as for the conjugation of benzoic acid, but while the small capacity of the organism to synthesize glycine does not materially limit the production of hippuric acid, it does definitely delay the synthesis of phenylaceturic acid. Thus, on supplying glycine exogenously, the production of phenylaceturic acid can be increased over 400 per cent. The speed of the conjugation of gly-

cine with phenylacetic acid is much faster than with benzoic acid. The best explanation for this difference in speed is found in the assumption that the two conjugations are brought about by different enzymes which vary in their distribution and probably also in their concentrations. In the dog the enzyme which brings about the synthesis of hippuric acid is present apparently only in the kidney, whereas the enzyme which causes the union of phenylacetic acid with glycine is present in other tissues beside the kid-

TABLE IV  
*Factors Influencing Rate of Conjugation of Phenylacetic Acid*

Date	Excretion of phenylacetic acid as						Remarks	
	Glycuronic acid mono- phenylacetate			Phenylaceturic acid				
	6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.		
1931	gm.	gm.	gm.	gm.	gm.	gm.		
Jan. 7*	0.50	0.60	0.35	0.47	0.78	1.10	5 gm. phenylacetic acid	
" 10	0.31	0.19	0.17	2.17	1.86	0.37	5 " " " and 15 gm. gelatin	
" 14	0.76	0.53	0.76	0.70	0.63	1.26	10 gm. phenylacetic acid	
June 24	0.50	0.50		0.55	0.50	1.87†	5 " " " 15 gm. gelatin 12 hrs. later	
	0.12	0.12	0.11	0.06	0.06	0.09	Routine diet	
Feb. 16‡	0.39	0.33		0.47	1.77		5 gm. phenylacetic acid; 10 gm. gelatin 6 hrs. later	

\* Dog 6, weight 10 kilos.

† Excretion in third 6 hour period.

‡ Dog 10, weight 9 kilos.

ney, for, as will be shown in a subsequent paper, a nephrectomized dog can still synthesize phenylaceturic acid. Whether glycuronic acid combines faster with benzoic acid than with phenylacetic acid cannot be determined until the kidney's threshold for glycuronic acid monophenylacetate is known.

It is noteworthy that neither benzoic acid nor phenylacetic acid is excreted uncombined. When these compounds are introduced into the body, they are presumably fixed, and their liberation and excretion is brought about by the processes of conjugation. If

it is possible to supply a conjugating component from exogenous sources as in the case of feeding glycine, the rate of liberation is greatly increased. This is clearly illustrated by the following experiment. On feeding 5 gm. of phenylacetic acid, the rate of excretion was about 1 gm. in 6 hours for two consecutive periods. By feeding gelatin at the beginning of the third 6 hour period, the rate of excretion was increased to 2 gm. or, in other words, doubled (Table IV).

The view that conjugation is a liberating process is of particular significance in regard to the question of detoxication. It raises the question whether the prime object of conjugation may not be to set the drug free from the tissue to which it is presumably attached rather than to render it innocuous.

#### SUMMARY

The syntheses of hippuric acid and glycuronic acid monobenzoate were studied from the standpoint of the law of mass action. It was concluded that these processes are intrinsically the same both in man and in the dog, and that the quantitative differences in the output of the two conjugated products depend on the factors which influence a reversible reaction.

The conjugation of phenylacetic acid was similarly studied by applying the principles of the law of mass action.

The hypothesis is presented that a compound like benzoic acid when introduced into the organism becomes fixed and that its liberation is brought about by the mechanism of conjugation.

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## THE SPONTANEOUS OXIDATION OF DIALURIC ACID

### III. THE OXIDATION OF AMINO ACIDS BY DIALURIC ACID\*

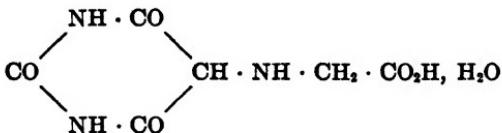
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Strecker (1) was the first to examine the action of alloxan on amino acids. He found that on warming a solution of alloxan with a solution of leucine, isovaleraldehyde and carbon dioxide were produced, and that alanine, when treated in the same way, gave acetaldehyde and carbon dioxide, while glycine gave no aldehyde but did give carbon dioxide; in all three cases the liquid assumed the color of murexide. No quantities were given in Strecker's paper, and he identified his products by qualitative tests.

Piloty and Finkh (2), in their paper on the constitution of murexide, describe the interaction of alloxan and glycine when concentrated solutions are mixed at 80°. In these circumstances, the color of murexide is produced, carbon dioxide is evolved, and on rapid cooling, a crystalline product is obtained which has the color of murexide. This product they describe as glycine purpurate. When, instead of being cooled, the mixture was heated until the color of the murexide had disappeared, an insoluble, amorphous substance was deposited, and the mother liquor yielded a yellow, crystalline solid, which they describe as uramiloacetic acid.



\* This manuscript is part of a dissertation presented to the Faculty of the Graduate School of the University of Cincinnati in May, 1931, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

This study was made possible by fellowships maintained by Professor J. U. Lloyd and Mr. Charles G. Merrell, Cincinnati.

Piloty and Finkh made no reference to the production of formaldehyde.

The apparently abnormal behavior of glycine towards alloxan led Hurtley and Wootton (3) to repeat the earlier experiments. In the first place, they confirmed the production of isovaleraldehyde from leucine and of acetaldehyde from alanine; they also tried the action of alloxan on  $\alpha$ -aminobutyric acid, and were able to show that propylaldehyde was produced. When molecular proportions of alloxan and glycine were heated in concentrated solution, no formaldehyde was produced, but they obtained the amorphous substance, and the uramiloacetic acid of Piloty and Finkh. When molecular proportions of alloxan and glycine in dilute solutions were distilled, formaldehyde was easily recognized in the distillate. Under these conditions, Piloty and Finkh's amorphous substance was not obtained. The liquid, which became purple soon after mixing, lost its purple color and yellow crystals identical with Piloty and Finkh's uramiloacetic acid were deposited on cooling. It is the opinion of Hurtley and Wootton that in the case of the concentrated solutions used by Piloty and Finkh, some of the uramiloacetic acid, or the uramil derived from it, had condensed with dialuric acid and formaldehyde to form the amorphous substance, thereby concealing the presence of formaldehyde. The dialuric acid was produced by the reduction of a part of the alloxan during the oxidation of the amino acid.

Hurtley and Wootton also found that dimethylalloxan oxidizes an  $\alpha$ -amino acid to the next lower aldehyde and yields tetramethylmurexide. In addition to the amino acids mentioned before, they tried the action of alloxan on tyrosine, tryptophane, cystine, and on glucosamine; all of these give a strong murexide color.

Traube (4) found that alloxan likewise oxidizes anilinoacetic acid to benzaldehyde and carbon dioxide, the solution becoming red. Besides alloxan, isatin, *p*-benzoquinone, and toluquinone were shown to oxidize the amino acid to aldehyde. Fatty aromatic amines, for example benzylamine, were in like manner oxidized to aldehydes by alloxan and isatin. Purely fatty amines, for example isoamylamine, were not oxidized by alloxan.

In view of the ease with which dialuric acid is oxidized to alloxan (5, 6) and with which it is obtained from alloxan on reduction, this portion of the study was conducted to discover whether amino

acids could be oxidized by the use of dialuric acid itself. The reaction promises to be one of considerable biochemical interest.

### Method

The amino acids used were glycine, alanine, valine, glutamic acid, and phenylalanine. The amount of amino acid employed in an experiment was usually 0.5 gm. except for one 0.25 gm.

TABLE I  
*Oxidation of Amino Acids by Dialuric Acid*

Amino acid	Weight of amino acid gm.	Weight of dialuric acid gm.	pH	Temperature °C.	Time hrs.	Amount of CO <sub>2</sub> absorbed gm.	Amount of NH <sub>3</sub> absorbed gm.	Oxidation per cent
Glycine.....	0.5	0.1	7.0	24.5	2.0	0.0046	0.00172	1.50
" .....	0.5	0.1	7.0	23.8	1.66	0.0042	0.00161	1.40
" .....	0.5	0.005	7.4	25.0	5.83	0.0073	0.00281	2.45
" .....	0.5	0.002	7.0	24.0	5.0	0.0011	0.00039	0.34
Alanine.....	0.5	0.1	7.0	24.9	2.0	0.0041	0.00153	1.60
" .....	0.5	0.1	7.4	25.4	5.66	0.0057	0.00219	2.30
" .....	0.5	0.005	7.6	25.3	5.0	0.0024	0.00091	0.96
Valine.....	0.5	0.01	7.4	23.7	5.0	0.0046	0.00324	4.51
" .....	0.5	0.01	7.0	25.1	6.0	0.0042	0.00295	4.11
Glutamic acid.....	0.5	0.01	7.0	24.7	5.25	0.0045	0.00174	3.02
" " .....	0.5	0.01	7.4	25.0	5.08	0.0066	0.00255	4.42
" " .....	0.5	0.01	7.6	25.5	6.25	0.0036	0.00139	2.41
Phenylalanine.....	0.25	0.01	7.4	24.9	6.5	0.0035	0.00132	5.30
" .....	0.5	0.01	7.6	25.2	6.0	0.0052	0.00196	3.93

sample of phenylalanine and the amount of dialuric acid varied from 0.002 gm. to 0.1 gm. according to the experiment (see Table I).

The reactions were measured by passing a stream of air, free from carbon dioxide and ammonia, through a mixture of the amino acid and dialuric acid, in water solution, at room temperature. The amino acid was dissolved in water (usually about 5 cc.) with the aid of 0.01 N HCl and 0.01 N NaOH, according to the solubility

of the acid, and the solution neutralized to the desired pH with these two solutions. An equal volume of buffer solution (0.5 M  $\text{KH}_2\text{PO}_4 + 0.5 \text{ M NaOH}$ ) of the same pH was added and the solution placed in the reaction chamber, a large test-tube of about 60 cc. capacity. The dialuric acid was added to this solution in solid form and the mixture placed in the reaction train. This was arranged as follows: A stream of air was passed through two bottles containing concentrated KOH solution to remove carbon dioxide, then through a bottle of concentrated  $\text{H}_2\text{SO}_4$  to remove ammonia and moisture. From this the air passed through the mixture of amino acid and dialuric acid. It then passed through two large tubes, the first containing 25 cc. of 0.01 N  $\text{H}_2\text{SO}_4$ , the second, 50 cc. of 0.01 N  $\text{H}_2\text{SO}_4$ , to absorb the ammonia given off; then through a wash bottle of concentrated  $\text{H}_2\text{SO}_4$  to remove moisture and then through two carbon dioxide absorption towers filled with ascarite (sodium hydrate asbestos). The carbon dioxide towers were weighed before and after each experiment. The  $\text{H}_2\text{SO}_4$  solutions in the two ammonia absorption tubes were titrated at the end of an experiment with 0.01 N NaOH, alizarin being used as an indicator. As stated above, the reaction chamber was at the temperature of the room. The air was passed through the apparatus at a rate of about 40 bubbles per minute.

The data and results are shown in Table I.

#### Results

Table I shows comparatively the amounts of carbon dioxide and ammonia given off by different amino acids at different pH values, different concentrations of dialuric acid, etc.

During the experiments, the solutions began to turn pink shortly after the dialuric acid had been mixed with the amino acid, and this color increased slightly as the reaction progressed, but never reached any great intensity, due to the minute quantities of dialuric acid added. The color was strongest in those cases where the largest amount of dialuric acid was used, *i.e.* 0.1 gm. This color was due to the formation of murexide, and persisted throughout an experiment. After the completion of an experiment, the solution was allowed to stand for several hours, the color faded from the liquid, and a pinkish sediment was found in the bottom of the tube. This was assumed to be the salt of the amino acid

and purpuric acid as described by Piloty and Finkh. The sediment was not examined.

In the experiments where glycine was used, formaldehyde was found to be present in the solution after oxidation. Its presence was shown by the  $\text{FeCl}_3$ -milk- $\text{H}_2\text{SO}_4$  test. All of the solutions showed tests for aldehydes by the Schiff reaction after oxidation. In the case of phenylalanine, the solution smelled strongly of hyacinths, this odor being characteristic of phenylacetaldehyde.

The course of the reaction appears to be as follows: The dialuric acid is oxidized by the oxygen of the air to alloxan, alloxan then acts on the amino acid, setting free ammonia and carbon dioxide, and making the aldehyde of the next lower acid. The alloxan is reduced in the process to dialuric acid and takes up oxygen again and so repeats the oxidation. Some of the alloxan unites with part of the dialuric acid to form alloxantin, which, with the ammonia, forms murexide. Proof that the solution contains alloxantin has been obtained by testing with barium hydrate, the characteristic violet color being obtained.

#### SUMMARY

Alloxan, formed by the autoxidation of dialuric acid, acts upon amino acids, setting free from them ammonia and carbon dioxide, and making the aldehyde of the next lower acid. The alloxan is reduced to dialuric acid in the process and takes up oxygen again and so repeats the oxidation.

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## SYNTHESIS OF THE HEXURONIC ACIDS

### I. THE SYNTHESIS OF *dl*-GALACTURONIC ACID FROM MUCIC ACID\*

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#### INTRODUCTION

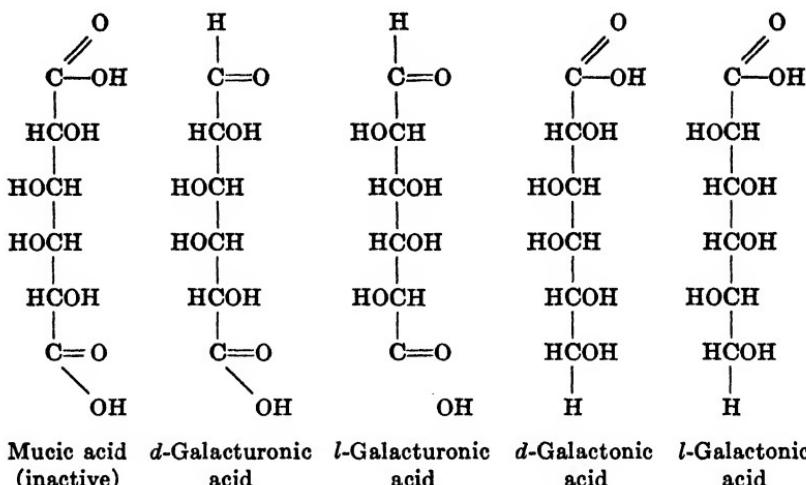
In the course of Fischer's classical researches in the sugar group, the oxidation of the aldoses to their dicarboxylic acids and reduction of these acids to the corresponding aldonic acids constituted significant steps in the elucidation of the stereochemical relationships of the sugars.

While developing a proof for the stereochemical configuration of mucic acid, which was regarded correctly by van't Hoff as one of the inactive systems of the ten theoretically possible dicarboxylic 6-carbon sugar acids, Fischer (1, 2) noted that when the lactone of mucic acid was reduced to *dl*-galactonic acid, an intermediate acid was formed in the course of the reduction which he called "Aldehydeschleimsäure."

Fischer pointed out that since complete reduction of the lactone of mucic acid yields *d*- and *l*-galactonic acid, the intermediate "Aldehydeschleimsäure" should also consist of a *d* and an *l* component.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

These relationships are illustrated structurally by the following formulæ:



Fischer's "Aldehydeschleimsäure"

The existence of the racemic "Aldehydeschleimsäure," as an intermediate acid formed in the course of the complete reduction of mucic acid lactone to *dl*-galactonic acid was inferred by Fischer on the basis of the following. He observed that, whereas the lactone of mucic acid did not reduce Fehling's solution, as it was being reduced, the reducing value of the reaction mixture increased to a maximum and then decreased.

Although Fischer stated that he intended to isolate the acid and characterize it more definitely,<sup>1</sup> he did not report further work on it in the course of his extensive studies in the carbohydrate group. In 1908, Mandel and Neuberg (3) reported that the Fischer "Aldehydeschleimsäure" gave the naphthoresorcinol test developed by Tollens for aldehyde and ketonic acids.

Apparently no one has attempted to isolate the intermediate

<sup>1</sup> Fischer (1) states, the lactone of mucic acid, in contrast to mucic acid, can be reduced readily, to form an aldehydic acid, which can be detected by its behavior toward Fehling's solution. The experimental studies involved are in progress. Later (2) Fischer states, the first phase of the reduction of the lactone of mucic acid yields, as has been mentioned before, an aldehydic acid whose presence can be detected with Fehling's solution.

sugar acid formed in the course of the reduction of the lactone of mucic acid, for this gap in the sugar group was regarded of sufficient importance to warrant a special comment (4) and furthermore, the comprehensive supplementary volume of Abderhalden (5) makes no reference to this acid.

On the other hand, the *d* form of galacturonic acid, one of the components of Fischer's "Aldehydeschleimsäure" was isolated in the pure state for the first time by Ehrlich, who found it to be one of the chief components of the pectin in the root of the sugar beet (6). Since then Ehrlich and his coworkers have isolated it from the pectin of other plant tissue (7). In 1923, Kiliani reported that he had isolated the *l* form of galacturonic acid by the oxidation of *d*-galactonic acid with nitric acid (8), but subsequently had the grace to refute this work and admit that an error had been made (9). Ohle and Berend reported the synthesis of *d*-galacturonic acid by the oxidation of diacetone *d*-galactose with potassium permanganate, but they were unable to isolate the free acid in a crystalline form (10).

Recently the aldehydic sugar acids have attained a preeminent position in biochemical and physiological researches due to their almost universal occurrence in both the plant and animal organism (5). Because of the great demand for relatively large quantities of these acids we have been engaged in the difficult task of developing satisfactory procedures for the preparation of the naturally occurring uronic acids (11) and also in a study of the properties of these interesting and important members of the sugar group (12). Since an inevitable destruction of the uronic acids occurs in the course of their preparation from natural sources (12) it was deemed advisable to explore the possibility of synthesizing them from the corresponding dicarboxylic sugar acids. The success that we have had with the synthesis of *dl*-galacturonic acid has led us to attempt the synthesis of other members of the group. In a forthcoming publication we will report on the synthesis of *dl*-alluronic acid from allomucic acid.

#### EXPERIMENTAL

The method of synthesizing *dl*-galacturonic acid reported in this paper was formulated along the lines originally laid down by Fischer (1, 2) in his experiments on the reduction of mucic acid

lactone to *dl*-galactonic acid. However, it was found advisable to introduce certain important modifications in the experimental procedure. We were able to establish a definite intermediate end-point in the course of the reaction, so that a good yield of the pure *dl*-aldehydic acid could be obtained.

*Preparation of Mucic Acid*—The mucic acid was prepared by the oxidation of lactose under the conditions of Kent and Tollens (13) and then carefully washed with distilled water to remove all traces of nitric acid. The acid employed in this investigation had a melting point of 220°. It was completely soluble in 60 parts of boiling water, and entered into solution without discoloration.

*Preparation of Mucic Acid Lactone (1, 2)*—50 gm. of mucic acid obtained by the above procedure were dissolved in 3 liters of boiling water contained in a large porcelain evaporating dish. The solution was then concentrated rapidly over a free flame to approximately 700 cc., whereupon it was cooled rapidly to 5° and filtered on a Buchner funnel. The clear filtrate contained the monolactone of mucic acid and mucic acid in the ratio of 10 parts of lactone to 1 part of acid. This ratio was determined by an alkalimetric titration. The actual isolation of the lactone is not performed. This is not a hindrance for the objective in view, since the solution thus obtained can be reduced directly.

*Reduction of the Monolactone of Mucic Acid (1, 2, 14)*—The aqueous solution of the lactone<sup>2</sup> was introduced into a 1 liter beaker, surrounded by an ice bath, and cooled to 5°. While the solution was being stirred vigorously, 300 gm. of 2.5 per cent sodium amalgam<sup>3</sup> were added during the course of 20 minutes. Dilute sulfuric acid (0.2 N) was added to the lactone solution throughout the entire course of the reduction, as recommended by Fischer and Kiliani in their procedure for the reduction of saccharic acid lactone to *d*-glucuronic acid (15). After the reaction

<sup>2</sup> From 50 gm. of mucic acid, about 10 gm. of the acid precipitates out in the concentration process. Of the remaining 40 gm. 5 gm. of mucic acid are in solution and the rest is present as the lactone.

<sup>3</sup> The ratio of sodium, in the form of amalgam, to lactone was 1.8 mols to 1.0 mol, for this ratio, under the conditions used, gave to the reaction product a maximum reducing power as determined by its action on Fehling's solution. This ratio may be increased in favor of the sodium but this involves the accompanying danger of carrying the reduction to the aldonic acids which are difficult to separate from the aldehydic acids.

had ceased (10 to 15 hours) the solution was decanted from the residual mercury. The filtrate was then cooled to 5° and basic lead acetate added carefully until no further precipitation took place.<sup>4</sup> The precipitate was then filtered off rapidly on a Buchner funnel and washed with 500 cc. of ice-cold water. It was then suspended in 400 cc. of ice-cold water and again filtered off at the pump. The precipitate, considerably freed from water-soluble salts, was suspended in a finely divided form in 1 liter of water and decomposed with hydrogen sulfide. The lead sulfide was removed by filtering on an asbestos mat and the filtrate aerated to eliminate the residual hydrogen sulfide remaining in solution. At this stage it is of importance to have the solution free from lead. It was noticed in a number of trials that lead is often present in the filtrate and that it must be removed by additional treatments with hydrogen sulfide.

The aerated filtrate was subsequently neutralized with an excess of barium carbonate (100 gm.), heated on a water bath at 70° for 30 minutes, and filtered. The filtrate was concentrated at 40° and 15 mm. pressure to a volume of 75 cc. Carbon dioxide was then passed through the solution for 5 hours. After this treatment the solution was placed in an ice chest for 2 to 3 days. This procedure eliminates any barium combined with the sugar acid in the form of the so called metal saccharates. The solution was centrifuged to remove the precipitated barium carbonate, and then poured into 800 cc. of 95 per cent ethyl alcohol, while the latter was being stirred vigorously. After standing for  $\frac{1}{2}$  hour the precipitated barium salt was filtered on a Buchner funnel, sucked dry, and redissolved in 100 cc. of water. It was then reprecipitated with 4 volumes of 95 per cent alcohol,<sup>5</sup> filtered, and washed successively with hot 95 per cent ethyl alcohol, hot absolute alcohol, and finally with absolute ethyl ether. The barium salt obtained was partially dried over calcium chloride in a vacuum desiccator at room temperature and then completely dried in a vacuum oven under 4 mm. pressure and a temperature of 50°. Numerous trials indi-

<sup>4</sup> This requires from 700 to 800 cc. of a basic lead acetate solution prepared according to the Association of Official Agricultural Chemists as given by Browne (16).

<sup>5</sup> Reprecipitation of the barium salt is necessary to remove any barium acetate that may be present.

cated that a yield of 15 to 20 gm. of *dl*-barium galacturonate are obtained from 50 gm. of mucic acid. This yield, calculated on the basis of mucic acid used, no recovery being assumed, is from 24 to 32 per cent of the theoretical. Allowing for a 20 per cent recovery of mucic acid during the preparation of the lactone, the yield is from 35 to 40 per cent of the theoretical.

*Constants*—Theory demands for  $(C_6H_8O_7)_2 Ba$ , 26.3 per cent barium. Found, 26.7 to 27.0 per cent Ba. Optical rotation in water  $[\alpha]_D^{25} = 0.0^\circ$ . The *dl*-barium galacturonate does not have a sharp melting point, even when pure, but begins to decompose at 180°. In this respect it is comparable to the pure *d*-barium salt prepared from citrus pectin (11).

*Isolation of dl-Galacturonic Acid from Barium Salt*—22 gm. of *dl*-barium galacturonate were dissolved in 500 cc. of water and subjected to an additional purification by heating at 50° in the presence of 5 gm. of blood charcoal and 5 gm. of kieselguhr. After filtering, the solution was cooled to 20°. Ethyl alcohol (95 per cent) was then added to produce a permanent turbidity, 100 cc. being necessary. While the solution was being vigorously agitated, 425 cc. of 0.2 N sulfuric acid were added during the course of  $\frac{1}{2}$  hour. 10 gm. of blood charcoal and 5 gm. of kieselguhr were added to the solution, still neutral to Congo red, which was then heated to 50° and filtered through an asbestos mat at the pump. The clear colorless solution was concentrated at 40° and 15 mm. pressure to a volume of 170 cc. The concentrate was then poured slowly into 400 cc. of 95 per cent ethyl alcohol to precipitate the undecomposed barium salt. It is advisable to use slightly less than the theoretical quantity of acid required so as to minimize lactone formation. The unchanged barium salt was filtered off, dried in a vacuum, and weighed. The clear filtrate was again concentrated at 35° under 15 mm. pressure to a volume of 75 cc. The concentrate was then filtered into a crystallizing dish through a mat of asbestos. After seeding with a crystal of the free *d* acid, which is desirable but not necessary, the dish was placed in a vacuum desiccator. After exhausting the desiccator for 15 minutes it was sealed off and the contents of the dish allowed to crystallize. Crystallization, if the experiment is successful, is usually complete in 2 to 3 days. The crystalline mass obtained was suspended in 30 cc. of 95 per cent ethyl alcohol and filtered

off on a hardened filter. The crystals were then washed with small successive portions of 95 per cent ethyl alcohol, absolute alcohol, and finally anhydrous ethyl ether. The crystalline *dl*-galacturonic acid was dried to a constant weight at room temperature in a vacuum desiccator containing phosphorous pentoxide. The *dl*-galacturonic acid crystallizes as the monohydrate in small microscopic needles. Yield, 4.5 gm. from 20 gm. of barium salt or 30 per cent of the theoretical. The yield can be increased to 50 per cent of the theory by reworking the mother liquor.

*Melting Point*—The substance sinters at 110°, turns red at 135°, and melts at about 156° with effervescence and decomposition. The melting point of the mixture of the pure *dl* acid with an authentic specimen of the *d* component prepared from citrus pectin showed no depression.

*Neutralization Equivalent*—1.0 gm. required 46.98 cc. of 0.1 N NaOH, whereas theoretically 1.0 gm. of  $C_6H_{11}O_6(COOH)$  requires 47.20 cc. of 0.1 N NaOH.

*Optical Rotation*— $[\alpha]_D^{25} = 0.0^\circ$  (in water).

#### DISCUSSION

The successful reduction of mucic acid lactone to *dl*-galacturonic acid depends greatly on the purity of the mucic acid used. Fischer (1, 2) repeatedly emphasized the necessity for pure starting materials in the course of researches in the sugar group, and it was found that success in the final crystallization of the free *dl* acid is only accomplished if this precaution is strictly observed. Consequently it is advisable to prepare the mucic acid from pure lactose or galactose rather than from the galactans obtained from the Western larch (*Larix americana*), for when prepared from this source, preparations are obtained that are difficult to purify.

In the reduction of the lactone with sodium amalgam it has been found more practicable to reduce the aqueous solution of the lactone rather than to attempt to isolate this intermediate product. The lactone has been isolated by Fischer (2) but the yield is so unsatisfactory as to make this step inadvisable.

Sodium amalgam was chosen as the reducing agent, because it is the only one of those that were tried which satisfied the needs of the reaction in question. Metallic calcium and catalytic re-

duction with the Adams-Voorhees catalyst at 2 atmospheres of hydrogen pressure gave no apparent reduction.

As a result of experience based upon thirty-two preparations of the *dl*-barium galacturonate from 50 gm. portions of mucic acid, we have found the following points of manipulative technique important enough to warrant mention. (1) Better yields are obtained if the reduction of the lactone is carried out in the presence of sulfuric acid, as Kiliani found with the lactone of saccharic acid (15). (2) Attempts to remove the unchanged mucic acid from the reaction mixture by forming the lead salt with neutral lead acetate led to appreciable losses of the aldehydic acid. (3) Barium carbonate in excess has proved more desirable than stoichiometrical amounts of barium hydroxide, for decomposition due to alkalinity is thereby reduced to a minimum. Furthermore the solution can be heated safely in the presence of an excess of barium carbonate to effect complete conversion of the lactone of the aldehydic acid to the barium salt. (4) The use of carbon dioxide to remove the barium bound as saccharate is necessary, for if this treatment is eliminated the barium salt may contain as high as 40 per cent barium, whereas theory demands 26.3 per cent.

The yield of the free *dl*-galacturonic acid from its barium salt corresponds to that reported by Link and Nedden (11) for the *d*-barium salt prepared from the polygalacturonide of citrus pectin. The resolution of the racemic galacturonic acid into the *d* and *l* components over the brucine salts will form the subject of a separate communication.

#### SUMMARY

Crystalline *dl*-galacturonic acid has been prepared by the reduction of mucic acid with sodium amalgam in a slightly acid solution. This synthesis was effected by conversion of the mucic acid into its monolactone which, upon reduction, yielded the racemic (*dl*-) aldehydic acid. This was isolated in the form of its barium salt, which was converted into the crystalline free acid by decomposition with sulfuric acid.

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## THE PREPARATION OF *d*-MANNURONIC ACID LACTONE\*

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(Received for publication, December 8, 1931)

### INTRODUCTION

The work of Nelson and Cretcher (1) has demonstrated that the alginic acid present in *Macrocystis pyrifera* is made up, in part at least, of *d*-mannuronic acid. At first these workers were not able to obtain either the free acid or its lactone in a crystalline condition, but after oxidation to a dicarboxylic acid they succeeded in obtaining the crystalline diamide and the diphenylhydrazide with properties and constants identical to the analogous compounds prepared from *d*-mannosaccharic dilactone. A crystalline cinchonine salt whose physical constants differed from those reported for both the cinchonine derivative of *d*-galacturonic and *d*-glucuronic acid was also obtained. Later Nelson and Cretcher (2) were able to convert this cinchonine salt into the crystalline lactone of *d*-mannuronic acid, so that its identity could be definitely established by the determination of its melting point and optical rotation. In a recent publication Bird and Haas (3) also reported that *d*-mannuronic acid was a constituent of the cell walls of the Laminariaceæ and of *Macrocystis pyrifera*.

Encouraged by the results obtained by Nelson and Cretcher, which incidentally were reported prior to the recent paper by Bird and Haas,<sup>1</sup> we were led to apply the technique that we have developed for the preparation of crystalline *d*-galacturonic acid

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<sup>1</sup> These authors apparently overlooked the second article of Nelson and Cretcher (2) wherein the identity of the acid is established with certainty.

(3, 4), and also *d*-glucuronic acid (unpublished), to the preparation of the lactone of *d*-mannuronic acid.

We have been able to prepare a pure barium hexuronate from the hydrolysis products of the alginic acid<sup>2</sup> present in *Macrocystis pyrifera*. The lactone of *d*-mannuronic acid can be prepared from this barium salt by means of a simple and direct procedure. The physical constants reported by Nelson and Cretcher (2) for *d*-mannuronic acid lactone have been confirmed by us. This compound, until recently inaccessible, can now be prepared with relative ease.

#### EXPERIMENTAL

The greater part of the improved procedure developed by one of us (4) for the preparation of *d*-galacturonic acid from the polygalacturonide obtained from citrus pectin is remarkably well suited for the preparation of the lactone of *d*-mannuronic acid from the alginic acid of *Macrocystis pyrifera*. The precautions that must be observed in the preparation of *d*-galacturonic acid apply also to the preparation of *d*-mannuronic acid. The hydrolysis of the alginic acid is conducted in a similar manner, with 2.5 per cent sulfuric acid.<sup>3</sup> After following the procedure recommended for the preparation of the barium salt of *d*-galacturonic acid to the stage where the filtered hydrolysate is to be concentrated, an important modification is introduced which is essential to obtain a barium salt free from unhydrolyzed polyuronides and decomposition products.

The hydrolyzed filtrate is concentrated in a vacuum at 50° to approximately 150 cc. and then clarified by heating at 80° for 15 minutes with charcoal and kieselguhr. After filtering, the solution is cooled to 4°, allowed to stand for 2 hours, and filtered again through the same filter bed. By adding 10 to 15 cc. of 95 per cent alcohol at 4° the unhydrolyzed polyuronides are fractionally precipitated. Some barium carbonate and other by-products are thereby also removed. The precipitated products are filtered off and then the fractional precipitation with alcohol is repeated.

<sup>2</sup> Prepared for us by the Kelco Company, San Diego, California.

<sup>3</sup> The method of Butler and Cretcher is radically different from ours. They hydrolyze with 80 per cent sulfuric acid at room temperature, for 5 days.

If the solution is still strongly colored at this stage another clarification with charcoal and kieselguhr is advisable. Finally the barium salts (mostly barium mannuronate) are completely precipitated by adding the clarified solution to 5 volumes of 95 per cent alcohol cooled to below 10°. The alcohol should be stirred vigorously during the precipitation. After standing for 2 hours, the barium salt is filtered and washed, following the same procedure recommended for the barium *d*-galacturonate. The barium salt isolated thereby is free from sugars, furan condensation products, and other obnoxious impurities. It is not known whether the barium salt is a mixture of pure *d*-mannuronic acid and *d*-glucuronic acid<sup>4</sup> or only *d*-mannuronic acid. However, no difficulty was experienced in obtaining barium salts with a barium content between 26.0 and 27.0 per cent (Pregl micro method). Theory requires for  $(C_7H_8O_7)_2Ba$ , 26.30 per cent barium. The barium salt of *d*-mannuronic acid does not have a sharp melting point, even when pure, but begins to decompose at 180°. In this respect it is comparable to the pure barium salt of *d*-galacturonic acid prepared from citrus pectin (4). Nelson and Cretcher ((1) p. 1920) reported that the barium salt that they obtained was precipitated as a heavy gum. With the procedure recommended by us the barium salt is precipitated in a finely divided flocculent state. It settles readily and can also be filtered satisfactorily. Nelson and Cretcher made note of the fact that the analysis of their barium salt indicated that it was mostly the barium salt of a 6-carbon aldehyde sugar acid, with small amounts of lactone and the barium salts of polyuronic acids which had escaped hydrolysis. They did not report a method for the complete purification of the

<sup>4</sup> Bird and Haas isolated two crystalline cinchonine salts from the hydrolysis products of *Laminaria*. One of these salts showed a melting point of 161°,  $[\alpha]_D^{20} = +154^\circ$ , figures that are only in approximate agreement with those reported by Nelson and Cretcher who give a melting point of 152°,  $[\alpha]_D^{20} = +113.6^\circ$ , for the cinchonine salt of *d*-mannuronic acid from the alginic acid in *Macrocystis pyrifera*. The other salt melted at 195–197° and showed  $[\alpha]_D^{20} = +112.8^\circ$ . The latter constants, on the other hand, are in fair agreement with those reported by Schmidt and Vocke (5) for what they claimed to be the cinchonine salt of glucuronic acid obtained from *Fucus serratus*. It is not possible to conclude from the evidence available at present whether the alginic acid from *Macrocystis pyrifera* contains both mannuronic and glucuronic acid or only mannuronic acid.

barium salt. Neither Nelson and Cretcher nor Bird and Haas report the yields or the analytical figures of the barium salts that they isolated. The difficulties experienced by Butler and Cretcher, mentioned above, are entirely eliminated by following the procedure given in this paper.

The barium salt that we obtained can be decomposed in the same way that the barium galacturonate is converted into the free galacturonic acid. The technique is identical, up to the crystallization stage, except that the addition of alcohol in the conversion process is eliminated and the temperature of the solution is kept at 50° to insure a more complete formation of the lactone. After the mannuronic acid has been liberated from the barium and subjected to the various clarifications, filtrations, and concentrations, the final syrup is taken up in 20 cc. of hot glacial acetic acid.<sup>5</sup> It is then filtered by suction through a bed of charcoal and a mat of asbestos on a hardened filter paper into a round bottom crystallizing dish and placed in a vacuum desiccator containing anhydrous calcium chloride. The desiccator is evacuated for about 1 hour and placed in a cool chamber. After about 4 days the crystallization is usually complete.

The crystals are filtered on a small Hirsch funnel, pressed free from the mother liquors, washed with four 5 cc. portions of glacial acetic acid, and finally with absolute alcohol and ether. They are then dried in a vacuum tube over P<sub>2</sub>O<sub>5</sub> for 8 hours at room temperature. We have experienced no difficulty in obtaining the pure lactone directly with the authentic melting point of 142–143° without finding it necessary to resort to a second crystallization. The lactone of mannuronic acid crystallizes in small prisms. If the melting point is below the accepted value, the lactone can be purified by dissolving it in a minimum amount of hot glacial acetic acid. The solution is heated for a few minutes on a water bath at 80° with a portion of freshly activated blood charcoal. It is then filtered slowly through a small Hirsch funnel containing a piece of hardened filter paper and a pad of fine asbestos. After standing for 12 hours at 10° the crystals will separate completely. The preparations are often colored a faint salmon-pink, even

<sup>5</sup> The use of glacial acetic acid for the formation and crystallization of the lactone of a uronic acid was first employed successfully with the lactone of glucuronic acid (6).

after a second purification. In spite of the slight discoloration they nevertheless show a constant melting point and the correct optical rotation,  $[\alpha]_D^{20} = +89.60^\circ$ .

### *Yield of d-Mannuronic Acid Lactone*

From 80 gm. of alginic acid with a uronic acid content of 68.00 per cent,<sup>6</sup> determined by the method of Dickson, Otterson, and Link (7), 30 gm. of Ba salt with a barium content of 26.95 per cent (Pregl micro method) were obtained. With alginic acids containing a higher uronic acid content the yield is correspondingly higher. 22 gm. of barium salt when converted into the free acid and taken up in glacial acetic acid yield approximately 3.0 gm. of *d*-mannuronic acid lactone. The yields reported represent the average obtained from ten preparations.

### SUMMARY

A direct method for the preparation of crystalline *d*-mannuronic acid lactone by the hydrolysis of the alginic acid from *Macrocystis pyrifera* is given. The alginic acid is hydrolyzed with 2.5 per cent sulfuric acid and the mannuronic acid first obtained as the barium salt. The barium salt is then decomposed with 0.2 N sulfuric acid and finally taken up in glacial acetic acid from which the lactone is crystallized directly.

In conclusion I wish to express my gratitude to Mr. M. J. Walsh, Vice-President of the Kelco Company, San Diego, California, for furnishing the alginic acid, and for his interest in this work. I am also indebted to my collaborator, Mr. Raymond Nedden, for carrying out some of the original hydrolysis experiments.—K. P. L.

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<sup>6</sup> The determination was conducted for 4½ hours. The polyuronides of the alginic acids hydrolyze very slowly, hence it is possible that complete hydrolysis of the alginic acid did not take place during this time and that the actual uronic acid anhydride content is higher.

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## THE OXIDATION OF METABOLITES

### I. THE MECHANISM OF THE OXIDATION OF $\alpha$ -HYDROXY FATTY ACIDS WITH POTASSIUM PERMANGANATE

By EDGAR J. WITZEMANN

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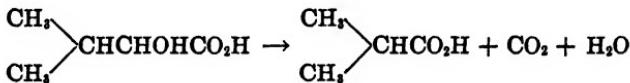
(Received for publication, June 1, 1931)

The mechanism of the oxidative breakdown of  $\alpha$ -hydroxy fatty acids has never been adequately studied from a chemical viewpoint and therefore any opinions as to the significance of  $\alpha$ -oxidation in biological processes that are current at the present time rest upon inadequate data. This paper constitutes a report on the oxidation of  $\alpha$ -hydroxy fatty acids and contains some data which clarify certain questions concerning the oxidation of these substances.

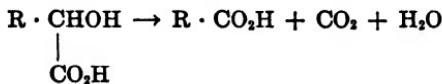
Although the oxidative breakdown of fatty acids in the organism has been the subject of many investigations, the prevailing views are for the most part not supported by adequate data from organic chemistry. This is strikingly true even in the case of butyric acid. Because of the importance of the acetone bodies in biology, the development on the biological side did not wait for the chemical facts concerning the oxidation of this substance. Theoretically the oxidation of butyric acid, for instance, can be initiated in a number of ways. Because of the lability of the hydrogen atoms on the  $\alpha$ -carbon atom, the organic chemist would select this as the point of first attack. As a matter of fact the  $\beta$ -carbon atom is almost universally regarded as the point of attack in biology. A careful examination of the question from the historical standpoint brings out the fact that this problem, even for the 4-carbon compound, was never fully rounded out on the chemical side. It was, so to speak, abandoned in midstream. It appears that in 1875 the fundamental problems in relation to this question were near solution in the case of the best known  $\alpha$ -hydroxy fatty acids,

namely lactic and  $\alpha$ -hydroxybutyric acids, but the workers were either drawn away to other fields of effort or perhaps regarded the problems as solved.

After the preliminary and pioneer work, by which the chemical constitution of the simple 3-carbon compounds was worked out, including especially lactic acid, organic chemists attacked higher members of the series. Among the workers thus engaged was Popoff. In 1871, having investigated the oxidation of isobutyric acid, with a mixture of sulfuric acid and potassium dichromate, he (1) reported that the oxidation of hydroxy fatty acids continues at the point at which it has been initiated; i.e., on the  $\alpha$ -carbon atom. He reported this also to be true with isobutyric acid and stated that this substance yields acetone and a little carbon dioxide. This work was continued and reported in various papers published in 1872 and 1873. In 1874 Ley and Popoff (2) published a paper on the oxidation of  $\alpha$ -hydroxy acids of the fatty series in which they said that, although it is known that lactic acid yields acetic acid and carbon dioxide upon oxidation, and although it would be possible to generalize from this result, before doing so further data ought to be assembled. They therefore oxidized  $\beta$ -methyl- $\alpha$ -hydroxybutyric acid with chromic acid and, as they expected, obtained isobutyric acid and carbon dioxide. The reaction was formulated as follows:



They then concluded that through this observation the above formulation of the oxidation of secondary hydroxy acids obtains a further confirmation and that it takes place according to the following reaction.



It is unnecessary to cite all of the circumstances and data but it is enough to say that this generalization came to be known as Popoff's rule.

In the following year Markownikoff (3) published a paper on the same subject. For 6 years he had been working on the oxidation of monohydroxy acids and at first believed Popoff's rule to be correct. He was, however, under the impression that this oxidation would take place with the intermediate formation of the corresponding  $\alpha$ -keto acid,  $\text{CH}_3\text{CHOHCO}_2\text{H} + \text{O} \rightarrow \text{CH}_3\text{COCO}_2\text{H} + \text{H}_2\text{O}$ , which upon further oxidation would yield the expected products, acetic acid and carbon dioxide. He was unable to obtain the intermediate compound<sup>1</sup> but obtained the expected end-prod-

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<sup>1</sup> This was achieved by Beilstein and Weigand (4) about 10 years later.

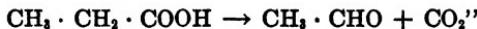
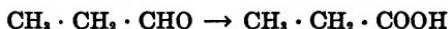
ucts. 2 years later Markownikoff had extended these studies to  $\alpha$ -hydroxybutyric acid, which he had obtained in the usual way from monobromo-butyric acid and had purified by repeated crystallization of the zinc salt. This salt decomposed with hydrogen sulfide yielded the free acid which was carefully oxidized with dichromate in sulfuric acid solution. Some unchanged  $\alpha$ -hydroxybutyric acid was recovered among the products but no  $\alpha$ -ketobutyric acid could be obtained. It was, however, clearly established that the remaining products were not propionic acid and carbon dioxide alone, but that the volatile acids consisted of a mixture of about two-thirds acetic acid and one-third propionic acid. This result was fully documented by repeating the oxidations and analyzing the silver and barium salts of the acids obtained.

Markownikoff states that the results had no particular interest for him until Ley and Popoff proposed their generalization. These authors had investigated two secondary hydroxy acids (lactic acid and  $\beta$ -methyl- $\alpha$ -hydroxybutyric acid) and set up their rule which when applied to a third acid,  $\alpha$ -hydroxybutyric acid, by Markownikoff failed. He stated that at the moment it was difficult to explain why the rule failed but that the data were incontestable. He said that although he regarded the rule as unproved he believed that in further work more instances supporting it would be found than exceptions to it. It was his plan to continue his quest for the  $\alpha$ -keto acids, but he published no further work on this or allied subjects, and seems to have been transferred to the study of Russian petroleum and minerals shortly after this.

Popoff at once wrote to Butlerow stating that Markownikoff's results were wrong due to the possible presence of  $\beta$ -hydroxybutyric acid in the acid subjected to oxidation; he reported that he had obtained two bromo acids in the bromination of butyric acid and that two hydroxy acids would therefore be obtained by the hydrolysis of the mixture of these. Butlerow reported this communication (5) but nothing further was published by Popoff, nor is this allegation against Markownikoff's work cited in Beilstein up to 1919. Moreover, Popoff's statement that two isomeric mono-bromo acids are obtained has failed to obtain support from other sources in the intervening 55 years, although this particular bromination is frequently carried out. Although Popoff's name has been forgotten, his rule (or rather its implications) has been accepted in biochemistry almost without a dissenting vote for two generations.

The chemistry of the oxidation of  $\alpha$ -hydroxybutyric acid received little or no further attention until 1908, when Dakin considered it in conjunction with his experimental study of the oxidation of ammonium butyrate with hydrogen peroxide. Knoop in 1904, from a study of the results obtained on feeding  $\omega$ -phenyl fatty acids, had observed that these acids characteristically lost 2 or a multiple of 2 carbon atoms from the side chain until they were reduced to benzoic or phenylacetic acid depending upon whether the side chain contained an even or an odd number of carbon atoms. In order to account for these results Knoop propounded his theory of  $\beta$ -oxidation. Moreover, he indicated that an analogous series of events probably occurs

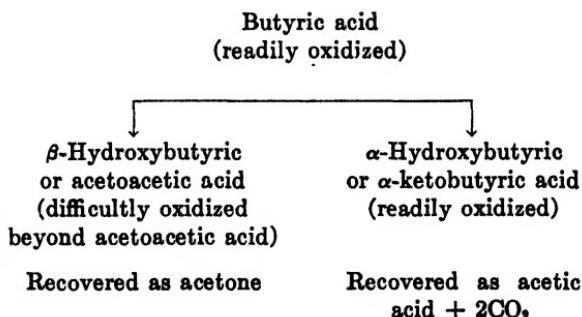
in the oxidation of fatty acids in the organism. At that time there were no chemical data concerning such an oxidation. This lack was, however, soon supplied by Dakin from his studies of the oxidation of the ammonium salts of fatty acids. In conjunction with this work Dakin also subjected  $\beta$ -hydroxybutyric acid to oxidation under the same conditions (6). He says, " $\alpha$ -Oxybutyric acid yields propionic aldehyde and propionic acid. The latter in part undergoes further oxidation with production of acetaldehyde and acetic acid which are partially oxidized further as already indicated:



In 1912 the writer prepared sodium  $\alpha$ -hydroxybutyrate and oxidized it with potassium permanganate, expecting to obtain results analogous to those previously obtained by the oxidation of sodium lactate in neutral solution; *i.e.*, propionic acid and carbon dioxide (7). He was not aware of the work of Markownikoff at this time and therefore thought that under these conditions Popoff's rule would be confirmed and that it would only be violated when the oxidation was carried out in alkaline solution, as it had been and is with lactic acid (8). However, the results obtained with sodium  $\alpha$ -hydroxybutyrate in neutral solution resembled those obtained with sodium lactate in the presence of free alkali. Circumstances prevented the continuation of this work and it was not until 1926 that a preliminary report could be made (9) in which it was shown that when sodium  $\alpha$ -hydroxybutyrate is oxidized with hydrogen peroxide in the presence of sodium phosphates only acetic acid and 2 molecules of carbon dioxide are formed. When potassium permanganate was used, the volatile acids obtained were a mixture of acetic and propionic acids. In both cases only propionic acid should have been obtained, whereas acetic acid was the sole or an important product. The results as reported were developed as a commentary to a previous paper (10) on the oxidation of sodium butyrate in the peroxide-phosphate system. The results of these studies have apparently not been understood, so that it seemed necessary to establish beyond a reasonable doubt the fact that  $\alpha$ -hydroxybutyric acid is not always or necessarily oxidized with the loss of 1 carbon atom at a time and that it

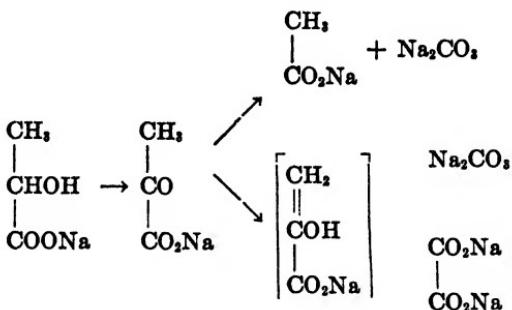
cannot on this account be regarded as excluded from consideration in the mechanism of fatty acid oxidation in the organism.

Dakin (11) had observed that in the oxidation of ammonium butyrate with hydrogen peroxide there is an oxidation on the  $\alpha$ -carbon atom. At that time this was the expected result and required no emphasis. The unexpected result was the formation of the ketone bodies and this was emphasized. Consequently, when the writer began working on the subject, both types of oxidation were to be expected. It was only as the details of the oxidative mechanism were studied that the unexpected result was again obtained. Whereas Dakin's work leads us to conclude that the normal results of biological oxidation, *i.e.* the loss of 2 carbon atoms at a time and the formation of the acetone bodies, could only be achieved by a  $\beta$ -oxidation, these new results indicated that the loss of 2 carbon atoms could be obtained even with that part of the butyric acid in which oxidation was initiated on the  $\alpha$ -carbon atom. In the work referred to above it was definitely proved that butyric acid in the phosphate-hydrogen peroxide system undergoes oxidation in two different ways. That is, oxidation is initiated on the  $\alpha$ -carbon atom as well as on the  $\beta$ -carbon atom. That the two types of products suffer a very different fate in this oxidation system was determined by control experiments. In tests made with  $\beta$ -hydroxybutyric acid and acetoacetic acid these compounds resisted oxidation in this system. Similar tests made with  $\alpha$ -hydroxybutyric acid showed this substance to be readily oxidized to acetic acid and 2 molecules of carbon dioxide. The results obtained may be readily summarized as follows:



The important facts developed in this work were first, the resistance of the products of  $\beta$ -oxidation of butyric acid to further oxidative attack, and second, the fact that  $\alpha$ -oxidation may be followed by the loss of 2 carbon atoms from the chain at one time. Both of these facts have considerable interest in biochemistry and opened up the whole question of the oxidation of fatty acids from another point of view. They suggest that perhaps  $\beta$ -oxidation in the organism, as generally conceived, is not obligatory to the loss of 2 carbon atoms from a fatty acid chain. In any case the results justified a thorough investigation of the mechanism of oxidation of fatty acids and their oxidative derivatives and this paper constitutes a first report in such a program.

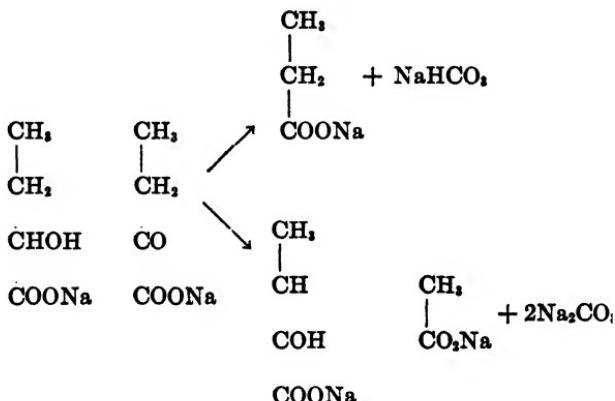
From the data previously obtained with lactic and  $\alpha$ -hydroxybutyric acids (9) it was concluded that the reaction, by which the chain is shortened by 2 carbon atoms instead of the expected number of 1, involves the intermediate formation of the  $\alpha$ -keto acids and that the ease of enolization determines the proportion of the products from the various types of cleavage. The reactions involved in the case of lactic acid may be visualized by the following formulas.



Owing to the relatively slight lability of the hydrogen atoms in the methyl group of lactic acid, considerable alkali is required to bring about appreciable labilization and enolization upon which the formation of oxalic acid in the oxidation depends.<sup>2</sup>

<sup>2</sup> It might be suggested that in this case the next product of oxidation of isopyruvic acid would be mesoxalic acid, and that it is impossible to say from which end of this molecule the carbon dioxide would be lost in giving rise to oxalic acid. This objection is not necessarily valid because mes-

In the case of  $\alpha$ -hydroxybutyric acid it was found that the methylene hydrogen is more easily enolized and that this takes place even in sodium phosphate buffer systems at pH  $\pm 7.3$ . Under these conditions oxalic acid does not appear among the products. The survival of oxalic acid in these oxidations depends upon the presence of enough free alkali to neutralize the second carboxyl group (12). Owing to the lability of the methylene hydrogen, the mechanism in this case would be represented thus:



The above constitutes a simple explanation of how products of  $\alpha$ -oxidation in the sense of Popoff and  $\beta$ -oxidation in the sense of Knoop may appear in the same reaction mixture. It also explains why Popoff's rule holds in some cases and why the results of Markownikoff were obtained.

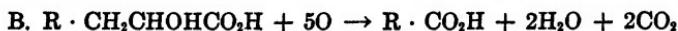
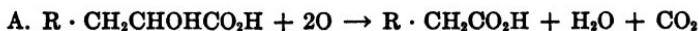
It is a fundamental question whether the same behavior is manifested by higher fatty acids. And it is especially important to know whether acids containing an odd number of carbon atoms behave as do the corresponding acids containing an even number of carbon atoms. It is always possible that the apparently simple homologous series of fatty acids may fall apart into two series in respect to some reaction, such as the mechanism of their oxidative breakdown. In fact, such suggestions have been made by various writers but need not be reviewed here.

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oxalic acid may not be formed normally, but it may be allowed to stand until further work establishes the facts. This objection does not, however, hold with  $\alpha$ -hydroxybutyric acid, as will be shown below.

In some preliminary oxidations of unsubstituted fatty acids with hydrogen peroxide and alkali phosphate as the catalyst, difficulties were encountered which indicated that the various questions involved would have to be answered separately. The various considerations that led to the adoption of the plan of attack used need not be detailed here. The first objective in this work was to determine what happens to  $\alpha$ -hydroxybutyric acid upon oxidation in the system used. The second objective was to determine whether or not the homologues of lactic and of  $\alpha$ -hydroxybutyric acids undergo oxidation in the same way that these acids do. In order to answer this question without ambiguity it was necessary to start with pure  $\alpha$ -hydroxy acids, oxidize them in such a way that the amount of oxygen used in the system could be accurately known, and also under conditions that would permit of determining the products of oxidation quantitatively. Potassium permanganate in neutral or faintly alkaline solution fulfilled the requirements for this reconnoitering study especially in view of the fact that the writer has had much experience in its use, particularly with lactic and  $\alpha$ -hydroxybutyric acids.

So far as the course of the reaction was concerned under these conditions, preliminary results showed that 85 per cent or more of the hydroxy acid molecules (with the possible exception of the higher hydroxy fatty acids used) underwent oxidation in accordance with one of the following two reactions:



These are the same reactions which had previously been observed to occur with lactic and  $\alpha$ -hydroxybutyric acids (9) and, of course, by Markownikoff.

The results obtained as summarized in Table I and as given in more detail in the experimental part are to be regarded as preliminary so far as the behavior of any one member of the series under various conditions is concerned. No attempt is made to report exhaustively on the various oxidations. The experiments reported represent typical results. So far as the series as a whole is concerned the results are about as definite as one can hope to obtain by the methods available at present.

The results in Table I show that beginning with lactic acid there is a shift from a preponderance of Reaction A to a preponderance of Reaction B; that is, from a loss of 1 carbon atom from the aliphatic chain to a loss of 2 carbon atoms with the higher members. The existence of this change was determined in three ways: by the amount of potassium permanganate reduced, by the amount of carbon dioxide formed, and by the composition of the volatile acids produced in the oxidation. For the higher members, i.e.,  $\alpha$ -hydroxyheptylic acid and the rest, the differences in the

TABLE I  
*Comparison of Products of Oxidation of  $\alpha$ -Hydroxy Acids*

No. of carbon atoms	Acid	Loss of 1 carbon atom*		Loss of 2 carbon atoms*	
		per cent <sup>t</sup>	per cent	per cent <sup>t</sup>	per cent
C <sub>3</sub>	Lactic acid	±84		±16	
C <sub>4</sub>	$\alpha$ -Hydroxybutyric	±47		±52	
C <sub>5</sub>	$\alpha$ -Hydroxyvaleric	±16		±84	
C <sub>6</sub>	$\alpha$ -Hydroxycaprylic	±12		±88	
.	$\alpha$ -Hydroxyheptylic	+ (?)		-100	
C <sub>8</sub>	$\alpha$ -Hydroxycaprylic†				
C <sub>10</sub>	$\alpha$ -Hydroxycapric†				
C <sub>12</sub>	$\alpha$ -Hydroxylauric†				
C <sub>16</sub>	$\alpha$ -Hydroxypalmitic†				
C <sub>18</sub>	$\alpha$ -Hydroxystearic†				

\* The writer wishes to emphasize the plus or minus signs. The results given throughout these papers are approximations. Anyone who has worked on problems of this sort will appreciate the difficulties encountered.

† With these acids side reactions, such as those described in Paper II, were developed to a sufficient extent to interfere effectively with the analytical procedure used for the first five members of the series. Other difficulties arose which made a slight change in conditions of the reaction seem wise.

physical constants by which the proportions of two adjacent members of the series could be determined, such as their volatility with steam, are too small to permit of accurate analysis of the mixtures. However, since the change in the type of reaction takes place before this point is reached, since the total volatile acid can still be determined, and since the amount of permanganate required for the two reactions has the ratio of 2:5, while the amount of carbon dioxide produced has the ratio of 1:2, there was no difficulty about determining approximately what was

happening. Fortunately the shift in the point of rupture occurred within the range in which all details of the oxidative process could be determined.

The results on the oxidation of the higher members of the series were complicated by another factor. The sodium salts of these acids are so easily hydrolyzed and the acid in question is so insoluble in water that it was necessary to have some excess alkali present before adding the potassium permanganate. This also accounts for the appearance of considerable amounts of oxalic acid with the higher acids in place of the carbon dioxide that could have been expected had this not been true. The results for these acids therefore come into closer relations with the results given in Paper II, in which moderate amounts of alkali were used, than they do with those given here. Since, however, they were largely exploratory they are included here.

The results obtained for this series of oxidations were entirely unexpected. It is true that Magnus-Levy and Meyer (13) suggested many years ago that possibly the formation of the acetone bodies is not obligatory in the oxidative breakdown of fats in the body, but that perhaps there is a facultative shift or change in the manner of oxidation of fatty acids under suitable conditions. There was no adequate experimental evidence to support the idea and its authors appear to have abandoned it since it is not given in the second edition of the work in which it was offered (14). The data here reported demonstrate the existence of a shift of the sort visualized by these authors, although of course it has only been demonstrated in *in vitro* experimentation.<sup>3</sup>

The data presented in this paper establish beyond a doubt that a shift occurs in the point of rupture of the carbon chain on passing from the 3-carbon member to the higher members of the  $\alpha$ -hydroxy fatty acid series. In lactic acid this rupture occurs mainly between the carboxyl and the  $\alpha$ -carbon atom. In  $\alpha$ -hydroxybutyric acid the chain is ruptured in part in the above manner

<sup>3</sup> The only other suggestion concerning a shift in the oxidative breakdown of fatty acids with which the writer is familiar is that of Hurtley (15), involving crotonic acid as an intermediate. Unfortunately no direct evidence is given for this ingenious suggestion, nor is any available at the present time so far as the writer knows. This does not, however, diminish the value of the suggestion.

and for the rest between the  $\alpha$ - and  $\beta$ -carbon atoms. With  $\alpha$ -hydroxyvaleric and  $\alpha$ -hydroxycaproic acids the rupture is largely of the latter type. With  $\alpha$ -hydroxyheptylic acid no evidence for the existence of the former type was obtained, probably due to difficulties in detecting small amounts of caproic acid in the presence of large amounts of valeric acid.

It is evident that the establishment of the existence of such a shift in the point of rupture raises more questions than it answers. These questions have received much consideration. It is believed that the answers to these questions will constitute an illuminating commentary upon the prevailing theory of  $\beta$ -oxidation of fatty acids, but that no revolutionary changes will appear. We shall find, however, that our present rather static view of fat oxidation will have been replaced by a dynamic one, just as our formerly static view of glucose in the organism is now replaced with a dynamic one.

#### EXPERIMENTAL

##### *Preparation of Materials*

The *dl*- $\alpha$ -hydroxy fatty acids were obtained by hydrolyzing the corresponding *dl*- $\alpha$ -bromo fatty acids which were obtained by brominating the corresponding normal fatty acids by the standard Hell-Volhard-Zelinsky method. The monobromo fatty acid bromide obtained in each case was hydrolyzed with a suitable alkaline substance and the hydroxy acid formed was separated from the solution in pure form as a salt.

In the case of  $\alpha$ -hydroxybutyric and  $\alpha$ -hydroxyvaleric acids, hydrolysis was carried out with barium carbonate, which had proved to be so convenient in the preparation of glycolic acid (16). The solution was then treated with sulfuric acid to remove barium, with moist, washed silver oxide in slight excess to remove bromides, and with hydrochloric acid to remove excess silver. The solution, free from barium, sulfuric acid, halogens, and silver, was then neutralized with zinc carbonate, evaporated, and cooled for the crystallization of the zinc salt.

In the case of  $\alpha$ -hydroxycaproic,  $\alpha$ -hydroxyheptylic, and  $\alpha$ -hydroxycaprylic acids hydrolysis of the corresponding  $\alpha$ -bromo fatty acid was usually brought about with sodium carbonate, although sodium hydroxide was used in some cases. In preparing

these acids it was unnecessary to purify the solutions before preparing the zinc salt. The salts were precipitated at once on adding zinc acetate to the faintly acid solution. In the case of  $\alpha$ -hydroxycaprylic acid it was impossible to obtain a normal zinc salt. This was not true with barium and so this and the remaining four higher fatty acids used were precipitated as the barium salts.

Since all of these  $\alpha$ -hydroxy acids are known and have been prepared by these methods and are described in organic chemical literature, it is not necessary to give further details in addition to the analytical data given in Table II.

TABLE II  
*Analyses of Salts of  $\alpha$ -Hydroxy Acids Used*

No. of carbon atoms	Salt	$H_2O$ of crystalli- zation	Zn or Ba	
			Found	Calculated
C <sub>3</sub>	Zinc lactate	2	23.31	23.40*
C <sub>4</sub>	" $\alpha$ -hydroxybutyrate	2	21.45	21.27*
C <sub>5</sub>	" $\alpha$ -hydroxyvalerate	2	19.36	19.44*
C <sub>6</sub>	" $\alpha$ -hydroxycaproate	None	19.88	19.87
C <sub>7</sub>	" $\alpha$ -hydroxyheptylate	"	18.08	18.40
C <sub>8</sub>	Barium $\alpha$ -hydroxycaprylate	"	29.98	30.17
C <sub>10</sub>	" $\alpha$ -hydroxycaprate	"	26.45	26.86
C <sub>12</sub>	" $\alpha$ -hydroxylaurate	"	24.00	24.21
C <sub>16</sub>	" $\alpha$ -hydroxypalmitate	"	19.88	20.39
C <sub>18</sub>	" $\alpha$ -hydroxystearate	"	18.30	18.68

\* Calculated for the hydrated salt.

#### *Methods of Analysis*

The carbon dioxide and oxalic acid formed were determined in the usual ways previously used.

The volatile fatty acids were distilled off after adding an excess of syrupy phosphoric acid.

The most convenient method of characterizing volatile fatty acids in these distillates when present singly or in pairs is by their volatility with steam in dilute solution. This method has recently been developed by Virtanen (17) and was also recently successfully used by the writer (18).

In order to get some additional characterization of the volatile

acid formed somewhat more extensive fractionations were also carried out. The data given in Table III were obtained with presumably pure acids. Stock aqueous solutions of propionic and butyric acids containing 0.25 gm. in 5 cc. were used. In the case of valeric, caproic, caprylic, and capric acids similar solutions containing 0.25 gm. of the free acid as the sodium salt in 10 cc. of water were used. With these the solution was diluted to about 200 cc., acidified with phosphoric acid, and distilled. The solutions of the former and the distillates of the latter were made up to 225 cc. and fractionated. The four 50 cc. fractions obtained and the undistilled residue were then titrated with 0.1 N sodium hydroxide. This is essentially the older method of Duclaux (19).

TABLE III  
*Results of Fractionation by Modified Duclaux Method*

Acid	0.1 N NaOH required						Acid present (calculated) gm.
	Frac- tion 1 cc.	Frac- tion 2 cc.	Frac- tion 3 cc.	Frac- tion 4 cc.	Resi- due cc.	Total alkali cc.	
Propionic, 0.50 gm.....	17.80	15.80	14.30	12.10	4.60	64.6	0.478
Valeric, 0.25 gm.....	11.70	6.75	3.40	1.40	0.90	24.15	0.246
Caproic, 0.25 " .....	12.35	5.45	1.70	0.45	1.30	21.25	0.246
Caprylic, 0.25 " .....	13.20	2.00	0.40	0.20	0.15	15.95	0.23
Capric*.....							

\* Fractionation of this and higher acids is unsatisfactory.

In using the method of Wiegner and Magasanik (20) as described by Virtanen the fatty acids were obtained as above; the solutions were made up to 240 cc. and of this 120 cc. were distilled off, the same flask and apparatus being used both in determining the constants of the apparatus and in examining unknown mixtures derived from oxidations of fatty acids.

The results of such distillations with 0.25 gm. of the acids in question and the constants obtained are given in Table IV.

*Oxidation of  $\alpha$ -Hydroxypropionic Acid (Lactic Acid)*—The results given in a previous paper (9) on the oxidation of sodium lactate with potassium permanganate in the absence of excess alkali are sufficient for this study. It was found that under these conditions about 84 per cent of the lactic acid used gave rise to

acetic acid, with the loss of 1 carbon atom, while the remainder presumably was completely disrupted, perhaps in part by the initial loss of 2 carbon atoms.

*Oxidation of  $\alpha$ -Hydroxy-n-Butyric Acid*—The results given here constitute an extension of data previously reported ((9) p. 216) on the nature and quantity of volatile acids formed in the oxidation of  $\alpha$ -hydroxybutyric acid with potassium permanganate. Only the results of the study of the nature of the volatile acids are given here in detail, since this is the point in dispute. Full details

TABLE IV  
*Results of Fractionation by Virtanen Technique*

Approximately 0.25 gm. of acid was used in each case.

Acid	0.1 N NaOH required		Acid present in 1st half	Acid present (calculated)
	1st half	Residue		
	cc.	cc.	per cent	gm.
Formic.....	12.50	38.30	24.6	0.233
Acetic.....	14.94	26.56	36.0	0.249
Propionic.....	19.15	13.35	58.8	0.240
Butyric.....	20.33	8.20	71.6	0.251
Valeric.....	19.90	3.95	83.4	0.243
Caproic.....	18.30	3.30	84.7	0.250
Caprylic.....	14.55	1.65	89.8	0.233
Capric.....	11.05	0.90	92.4*	0.206

\* Approximate results. Capric acid is so insoluble in water that this method is no longer of any value.

concerning the remaining products of this oxidation will be found in Paper II of this series.

5.17 gm. of zinc  $\alpha$ -hydroxybutyrate were treated with the requisite amount of sodium carbonate. To the filtrate which contained 3.50 gm. of  $\alpha$ -hydroxybutyric acid as the sodium salt 800 cc. of 0.1 M potassium permanganate were added. After reduction was complete, the manganese dioxide was filtered off. The volatile acids in the oxidation solution were recovered in the usual way by distilling with excess of syrupy phosphoric acid. One-half of the distillate was made up to 420 cc. and three fractions of 105 cc. were distilled off, leaving a residue of 105 cc. which was treated as a fourth fraction. These were diluted to

240 cc. and 120 cc. were distilled from each. These distillates and the residual portions required 0.1 N sodium hydroxide as follows:

*Fractionation of Unknown Mixture*

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
1st half, 120 cc.....	13.00 cc.	12.60 cc.	12.20 cc.	13.2 cc.
2nd " 120 "	12.80 "	13.25 "	13.90 "	17.2 "
	25.80 cc.	25.85 cc.	26.10 cc.	30.4 cc.
Acid in 1st half.....	50.3%	48.7%	46.7%	43.5%
Propionic acid.....	16.00 cc.	14.10 cc.	11.90 cc.	9.40 cc.
Acetic acid.....	9.80 "	11.75 "	14.20 "	21.00 "

Total propionic acid, 57.30 cc. or 47.5%

" acetic " 56.75 " " 52.5%

The amount of propionic and acetic acids present in these distillates was calculated by the method referred to above, by setting up simultaneous equations like the following and solving for A and P.<sup>4</sup>

$$A + P = 25.8 \text{ cc.}$$

$$0.366 A + 0.588 P = 13.00 \text{ cc.}$$

The results of these calculations are given in the last two lines of the tabulation above.

This procedure of separating the volatile acids into four fractions was adopted in order to provide a wider spread in the volatility constants for volatile acids other than acetic and propionic acids that might be present as impurities in the volatile products of oxidation. In order to get a check on the analysis of the above unknown mixture from both the qualitative and the quantitative

<sup>4</sup> In this pair and subsequent pairs of equations the data given in Table IV are used to calculate the amounts of two acids present in a distillate. The initial letters A, P, etc., represent acetic acid, propionic acid, etc., mentioned in the context. The first equation is for the entire distillate, in this case Fraction 1. The second equation is for the first half or 120 cc. Only 36.6 per cent of acetic acid is volatilized in the first half and 58.8 per cent of propionic acid, and therefore corresponding fractions of A and P are placed in the equation. For full details consult Virtanen's papers, especially that by Virtanen and Pulki (17).

standpoints a synthetic mixture of propionic and acetic acids was prepared such that it was estimated to contain propionic acid equivalent to 51.30 cc. of 0.1 N sodium hydroxide and acetic acid equivalent to 56.75 cc. of 0.1 N sodium hydroxide. This was diluted to 420 cc. and fractionated into four fractions exactly as above. These were diluted to 240 cc. and treated as above.

*Fractionation of Synthetic Mixture*

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
1st half, 120 cc.....	12.84 cc.	12.32 cc.	12.07 cc.	13.44 cc.
2nd " 120 " .....	12.61 "	12.82 "	13.58 "	17.57 "
	25.45 cc.	25.14 cc.	25.65 cc.	31.01 cc.
Acid in 1st half.....	50.4%	49.0%	47.0%	43.3%
Propionic acid.....	15.85 cc.	14.05 cc.	12.07 cc.	9.41 cc.
Acetic acid.....	9.60 "	11.09 "	13.58 "	21.60 "

Total propionic acid, 51.38 cc. or 48.4%  
" acetic " 54.87 " " 51.6%

The total volatile acid was only 106.2 cc., whereas the total desired was 108.1 cc. This would give rise to a small discrepancy in the results. In general the correspondence was very much better than was expected.

The ratio of the acetic and propionic acids present in the products of this oxidation is nearly 1:1 in this case. In the earlier data referred to above, the ratio could be determined only approximately and was thought to be about 1:2. As will be seen from the results given in Paper II of this series, the course of the oxidation of  $\alpha$ -hydroxybutyric acid is changed by slight changes in the conditions.

In the work of Markownikoff the delicate volatility method for assaying these acids available at present could not be used. He had to depend on the analysis of the silver and barium salts. In order to relate our experiments more adequately with this old work, it was decided to analyze the volatile acid mixtures by the same method of analysis used by Markownikoff. For this purpose another portion of 3.50 gm. of  $\alpha$ -hydroxybutyric acid was oxidized as described above. The volatile acids were recovered as usual. A portion analyzed by the volatility method

was found to consist of about 47.5 per cent propionic acid and 52.5 per cent acetic acid. The remainder was neutralized with barium carbonate by heating under a reflux condenser. The filtrate was evaporated to dryness and dehydrated at 110° to remove water of crystallization. The barium content of the dry, powdered salt mixture was then determined gravimetrically as barium sulfate. In this case 50.57 per cent barium was found (calculated for  $\text{Ba}(\text{CO}_2\text{CH}_3)_2$  53.70 per cent, found 53.47 per cent; calculated for  $\text{Ba}(\text{CO}_2\text{CH}_2\text{CH}_3)_2$  48.40 per cent, found 48.23 per cent). This means that about half of the acid is acetic and half propionic. Several other analyses were made with this material, as well as the volatile acids from other oxidations, and the results were the same within 0.1 of 1 per cent.<sup>5</sup>

In general, the results in hand show that under the conditions used about one-half of the  $\alpha$ -hydroxybutyrate is oxidized with the loss of 1 carbon atom and the remainder with a loss of 2 carbon atoms. Further evidence for this statement is found in Paper II.

*Oxidation of  $\alpha$ -Hydroxy-n-Valeric Acid*—From preliminary oxidations it was found that 0.50 gm. of  $\alpha$ -hydroxyvaleric acid as the sodium salt will reduce 116 cc. of 0.1 M potassium permanganate. Reaction A would require 56 cc., while Reaction B would require 140 cc., theoretically.

<sup>5</sup> The question will be raised as to why the content of acetic and propionic acids was not calculated algebraically from the weight of the mixed salts and the barium sulfate obtained. This was done first with the known mixture of acetic and propionic acids and then with the unknown mixtures. With the synthetic mixtures the results obtained by the volatility method agreed closely within 1 per cent with the composition of the mixture. On the basis of the barium content when the salts of the mixture were prepared and analyzed as described above, the propionic acid content was found to be 5 to 10 per cent greater than the calculated, and accordingly the acetic acid was 5 to 10 per cent too low. Similarly, calculations based on the barium content of the mixed barium salts of the unknown mixtures obtained by oxidation gave results about 10 per cent greater for propionic acid and 10 per cent lower for acetic acid than those given by the volatility method. Since there is no reason to believe that this can ever be a good quantitative method for the analysis of these salt mixtures, mathematical and other details have been omitted. The data as given constitute a confirmation of Markownikoff's work and also constitute good qualitative evidence in support of the other data given above for the composition of these mixtures.

A solution containing 0.50 gm. of  $\alpha$ -hydroxyvaleric acid as the sodium salt in 50 cc. of water was treated with 116 cc. of 0.1 M potassium permanganate. After reduction was complete, the manganese dioxide was filtered off and washed with water as usual. The filtrate was placed in a distilling flask arranged so that syrupy phosphoric acid could be added through a dropping funnel and so that the distillate could be collected while a current of carbon dioxide-free air was being circulated through the apparatus. The carbon dioxide liberated from the oxidation solution was collected in absorption bottles containing barium hydroxide solution. In this way the volatile acids and the carbon dioxide could be obtained simultaneously.

In two experiments 1.40 and 1.47 gm. of barium carbonate were obtained, respectively; *i.e.*, 0.312 and 0.328 gm. of carbon dioxide, or 83.8 and 88.1 per cent of the expected yield on the basis of Reaction B.

The distillate was made up to 240 cc. and 120 cc. were distilled over. The first half required 24.9 cc., the residual half 17.6 cc. of 0.1 N sodium hydroxide; total 42.5 cc.; *i.e.*, 58.6 per cent of the acid present was volatile in the first half of the distillate. In the other experiment the two fractions required 24.80 and 16.30 cc. of 0.1 N alkali, respectively, or 41.10 cc. altogether, and 60.2 per cent was volatile in the first half.

If the acid had been pure propionic acid as required by Reaction B, 58.9 per cent would have been volatile in the first half.

The volatile acid was recovered in both cases and fractionated in 50 cc. portions from a volume of 225 cc. The results obtained on titrating with 0.1 N NaOH were as follows:

Fraction 1	Fraction 2	Fraction 3	Fraction 4	Residual portion
cc.	cc.	cc.	cc.	cc.
11.65	9.85	8.00	6.10	4.70
12.20	10.00	8.10	6.15	4.05

These results resemble those given above for pure propionic acid except that the acidity of the residue is higher than it should be. This is due presumably to contamination with small amounts of acetic or formic acid formed as by-products of the oxidation.

On the basis of the alkali required for the first distillation, 42.5

and 41.1 cc., respectively, the acidity recovered corresponds to  $0.0118 \times 42.5 (41.1) = 0.501$  gm. (and 0.485 gm.) of  $\alpha$ -hydroxyvaleric acid accounted for. Since 0.50 gm. was used, this indicates that approximately 1 molecule of volatile acid was obtained for each molecule of the non-volatile acid oxidized.

It was desired to make more extensive fractionations of the volatile acids. A solution of 3.00 gm. of  $\alpha$ -hydroxyvaleric acid as the sodium salt was oxidized with 696 cc. of 0.1 M potassium permanganate. The volatile acids recovered as usual were made up to a volume of 600 cc. and fractionated in four parts of 150 cc. each. These were diluted to 240 cc. and fractionated in halves; 0.1 N alkali was required as follows:

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
1st half, 120 cc.....	38.70 cc.	31.40 cc.	24.80 cc.	18.50 cc.
2nd " 120 " .....	22.20 "	20.10 "	17.45 "	20.40 "
	60.90 cc.	51.50 cc.	42.35 cc.	38.90 cc.
Acid in 1st half.....	63.5%	60.9%	58.7%	47.4%
Butyric acid.....	22.65 cc.	8.6 cc.	-0.7 cc.	(0?)
Propionic " .....	37.25 "	42.9 "	42.35 "	19.7 cc.?

The above results were calculated by the method of Virtanen.

$$P + B = 60.9 \text{ cc.}$$

$$0.588 P + 0.716 B = 38.7 \text{ cc.}$$

Solving these simultaneous equations gives 22.65 cc. as the equivalent for the butyric acid present in Fraction 1. Fractions 2 and 3 gave 8.6 and 0.0 cc., respectively, for the butyric acid present. Fraction 4 was probably largely propionic and acetic acids, and when calculated on the assumption that

$$A + P = 38.90 \text{ cc.}$$

$$0.360 A + 0.588 P = 18.50 \text{ cc.}$$

gave 19.7 cc. as the equivalent for the propionic acid present.

From these results it appears that the butyric acid constitutes about 16 per cent of the volatile acids found, or about 22 per cent of that found in Fractions 1 to 3. This indicates that the oxidation takes place at least 16 per cent according to Reaction A and

not more than 84 per cent according to Reaction B so far as the volatile acids produced are concerned. The uncertainty is due to the influence of the products of side reactions.

The total volatile acid, 193.6 cc., corresponds to but 2.3 gm. of the 3.0 gm. of  $\alpha$ -hydroxyvaleric acid used in the oxidation.

*Oxidation of  $\alpha$ -Hydroxy-n-Caproic Acid*—3.72 gm. of the zinc salt were decomposed with 1.2 gm. of sodium carbonate. The filtrate was treated with 600 cc. of 0.1 M potassium permanganate. After the reduction of this, 50 cc. more were added 5 cc. at a time or until reduction was incomplete after standing at room temperature all night. Reduction was completed at 51° and the manganese dioxide was filtered off. The volatile acid was recovered in the usual way and made up to a volume of 750 cc. This was fractionated into five 150 cc. portions as usual, each of which was made up to 240 cc., fractionated in halves, and titrated with 0.1 N sodium hydroxide as usual.

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
1st half, 120 cc.....	61.50 cc.	43.70 cc.	28.30 cc.	16.00 cc.	6.70 cc.
2nd " 120 " ....	17.90 "	13.60 "	9.95 "	6.90 "	13.90 "
	79.40 cc.	57.60 cc.	38.25 cc.	22.90 cc.	20.60 cc.
Volatile acids in 1st half.....	77.4%	76.2%	73.9%	69.7%	32.5%

Fractions 1 to 4 were united and divided into three equal portions and then diluted to 240 cc. and fractionated in halves as before. In these 72.9, 73.2, 73.04 per cent of the acid present, respectively, were volatile in the first half. On the basis of the amount of potassium permanganate reduced, the main reaction could have been Reaction B. If but 1 carbon atom had been lost, valeric acid would have been formed. On the basis of the above results and on the basis of the fact that 71.6 per cent of butyric acid is volatilized in the first half and 84.7 per cent of the valeric acid, the following equations could be set up.

$$B + V = 65.45 \text{ cc.}$$

$$0.716 B + 0.847 V = 47.80 \text{ cc.}$$

$$I.e., \text{butyric} = 57.85 \text{ cc. or } 88.4 \text{ per cent}$$

$$\text{Valeric} = \frac{7.6}{65.45} " 11.6 " "$$

$$65.45 "$$

The total volatile acid recovered was equivalent to 218.4 cc. of 0.1 N sodium hydroxide; this corresponds to 2.88 gm. of  $\alpha$ -hydroxy-*n*-caproic acid (3.00 gm. used). The acid present in Fractions 1 to 4 was equivalent to 196.4 cc. of 0.1 N sodium hydroxide, *i.e.* 2.59 gm. of the acid used, and represents a recovery of valeric and butyric acids corresponding to 86.3 per cent of the  $\alpha$ -hydroxy-*n*-caproic acid used.

The carbon dioxide formed in the solution by oxidation was 1.64 gm. Assuming that each molecule of acid used gave rise to 2 molecules of carbon dioxide, 2.0 gm. should have been obtained. The amount obtained is therefore 82.0 per cent of that expected on the basis of the reaction given.

These results show conclusively that  $\pm 12$  per cent of the  $\alpha$ -hydroxycaproic acid was oxidized according to Reaction A and  $\pm 88$  per cent according to Reaction B on the basis of the volatile acids recovered.

*Oxidation of  $\alpha$ -Hydroxy-*n*-Heptylic Acid*—10.0 gm. of the zinc salt were decomposed with a slight excess of sodium carbonate. The filtrate was made up to 500 cc.

Of this 100 cc. ( $\equiv$  1.64 gm. of free  $\alpha$ -hydroxyheptylic acid) were treated with 5.87 gm. of potassium permanganate in about 300 cc. of water. After reduction was complete, the filtrate was diluted to 500 cc. Of this, 200 cc. yielded volatile acid which when fractionated in halves from a volume of 240 cc. required 30.70 cc. of 0.1 N alkali for the first half and 7.50 cc. for the remaining half; *i.e.*, 80.3 per cent is volatile in the first half. The main product expected was valeric acid of which about 83.4 per cent is volatile in the first half. The volatile acid recovered was equivalent to 1.40 gm. of the acid used; *i.e.*, 84.7 per cent was recovered as volatile acid.

During the distillation the carbon dioxide evolved was collected as usual. This, after subtraction of the carbon dioxide present in the original solution, was found to be 0.87 gm.; the calculated amount corresponding to Reaction B is 0.98 gm.; *i.e.*, a yield of 88.7 per cent.

The above oxidation was repeated with 3.5 times as much of the reagents, the requisite amount of 0.1 M potassium permanganate being used. The filtrate was made up to 1400 cc. Of this, 500 cc. were concentrated to about 225 cc., treated with excess of

syrupy phosphoric acid, and the volatile acids distilled off. The distillate was fractionated in two parts from a volume of 240 cc. The first half of the distillate required 90.40 cc. of 0.1 N alkali; the remaining half 22.40 cc.; i.e., 80.1 per cent was volatile in the first half; the volatile acid recovered corresponds to 80.7 per cent of acid used.

These volatile acids were recovered and fractionated into three equal fractions. Each of these was diluted to 240 cc. and fractionated in halves. Cc. of 0.1 N NaOH were required as follows:

	Fraction 1	Fraction 2	Fraction 3
1st half, 120 cc.....	58.80 cc.	19.80 cc.	6.20 cc.
2nd " 120 "	9.90 "	5.90 "	5.10 "
	68.7 cc.	25.7 cc.	11.3 cc.
Volatile acid in 1st half.....	85.6%	77.04%	54.7%

These results show that there was undoubtedly much valeric acid present as well as some caproic acid, as indicated by the results in Fraction 1. Fraction 2 contains some less volatile components. Part of this difficulty is due to the formation of acids with shorter chains and part of it is due to the slow volatility of unchanged  $\alpha$ -hydroxyheptylic acid.

In this case the carbon dioxide formed was also determined and was found to be 2.45 gm. or 70.8 per cent of the calculated yield on the basis of Reaction B. The oxalic acid present was found to be 0.986 gm. or 27.9 per cent of the calculated yield, assuming that each molecule of the acid oxidized yielded 1 molecule of oxalic acid. The data obtained indicate that the major part of the oxidation took place essentially according to Reaction B, except that some oxalic acid was formed in place of part of the carbon dioxide.

*Oxidation of  $\alpha$ -Hydroxy-n-Caprylic Acid*—Great difficulty was experienced in decomposing the zinc salt of  $\alpha$ -hydroxycaprylic acid with sodium carbonate. For this reason this salt and the salts of the remaining higher homologues were decomposed with hydrochloric acid and recovered by extraction with ethyl ether. After the extraction the ether was evaporated and the residual hydroxy acid after having been weighed was dissolved in sufficient

1.0 N sodium hydroxide. 5 gm. of the zinc salt of  $\alpha$ -hydroxy-caprylic acid yielded 4.0 gm. of the free acid. This, in the form of the sodium salt, was made up to about 400 cc. and treated with 13.1 gm. of powdered potassium permanganate, a little at a time, during 3 days. The last permanganate was reduced by warming the solution at 50°. The filtrate from the manganese dioxide was made up to 500 cc.

The carbon dioxide found was 0.668 gm.; *i.e.*, 30.3 per cent of the calculated yield on the basis of Reaction B.

The oxalic acid present was 0.40 gm.; *i.e.*, 17.8 per cent of the calculated yield, assuming that 2 carbon atoms from each molecule of acid used are converted into oxalic acid.

Of the volatile acids recovered 74.0 and 74.3 per cent, respectively, were volatile in the first 120 cc. when distilled from a volume of 240 cc. In these two determinations the acid recovered was 80.6 and 85.0 per cent, respectively, of the calculated amount.

In another oxidation 82.2 and 83.1 per cent of the acid in the distillate were volatile in the first half. The expected product was caproic acid of which 84.7 per cent is volatile in the first half. The results therefore show that the distillate is contaminated with impurities having a lower volatility, and it is impossible at present to determine how much caproic acid was formed. In this latter case only 72.0 per cent of the expected amount of volatile acid was recovered. Likewise, 40 per cent of the calculated amount of carbon dioxide was obtained. Oxalic acid was not determined.

The results of this oxidation show clearly that considerable oxidation takes place according to Reaction B, but that this result is complicated to an unknown extent by side reactions and difficulties of analysis.

*Oxidation of  $\alpha$ -Hydroxy-n-Capric Acid*—In a preliminary oxidation the carbon dioxide formed was found to be much less in amount than was expected. Tests showed that oxalic acid was present in considerable amounts. Oxidations with larger amounts of materials were then set up.

5 gm. of barium  $\alpha$ -hydroxycaprate were decomposed with hydrochloric acid. The free acid was recovered with ether. The 3.40 gm. of free acid obtained were converted into the sodium salt and the solution was made up to about 300 cc. To this were added 9.4 gm. of powdered potassium permanganate, a little at a time,

during 3 days. The last of the permanganate was reduced by warming the solution at 50°. After filtering off the manganese dioxide, the filtrate was made up to 500 cc. and analyzed as usual.

The carbon dioxide recovered was 0.47 and 0.45 gm. or 29.5 and 28.0 per cent, respectively, of the amount expected on the basis of the permanganate that was reduced; *i.e.*, Reaction B.

The oxalic acid present was found to be 0.56 and 0.535 gm. or 34.4 and 32.8 per cent, respectively, on the assumption that 2 carbon atoms from each molecule of fatty acid are converted into oxalic acid.

The volatile acids were recovered and distilled in halves from a volume of 240 cc. 76.6 and 74.6 per cent were volatile in the first half. If the acid had been pure caprylic acid, 89.8 per cent of it would have been present in the first half of the distillate.

In other oxidations 80.5, 84.0, and 84.2 per cent of the acid were present in the first half, showing that the results of this oxidation are somewhat variable. The volatile acids recovered constituted 83, 91, and 84 per cent of the expected quantity on the basis of the acid used.

Here again it is obvious that considerable oxidation takes place according to Reaction B, but the results are complicated by side reactions.

*Oxidation of α-Hydroxy-n-Lauric Acid*—10 gm. of barium α-hydroxylaurate were decomposed with hydrochloric acid. 6.8 gm. of crystalline free acid were recovered from the ether extract. This was dissolved with sufficient sodium hydroxide.

3.4 gm. of α-hydroxylauric acid in 300 cc. were rendered sufficiently alkaline to hold the salt in solution. To this, 524 cc. of 0.1 M potassium permanganate were added. After reduction was complete, the filtrate was found to contain 0.535 gm. of oxalic acid or 37.9 per cent on the assumption that each molecule of fatty acid yielded 1 molecule of oxalic acid.

The volatile acid obtained corresponded to 78.8 per cent of the acid used, but only 65.3 per cent of it was volatile in the first 120 cc. when it was distilled from a volume of 240 cc. Some globules of insoluble fatty acids floated in the distilling flask on top of the residual 120 cc. This fact alone made the volatility data anomalous.

The carbon dioxide recovered was 0.365 gm. which is 25.8 per cent of that expected on the basis of Reaction B.

It appears the Reaction B also occurred here but that the results are covered up by side reactions and by the anomalous volatility of the acids involved.

*Oxidation of  $\alpha$ -Hydroxy-n-Palmitic Acid*—Barium  $\alpha$ -hydroxy-palmitate was decomposed as the corresponding lauric acid salt and yielded 4.4 gm. of the crystalline acid. This was converted into the sodium salt and treated with 538 cc. of 0.1 M potassium permanganate as usual.

The colorless filtrate was found to contain 0.33 gm. of oxalic acid, which is 22.6 per cent of the theoretical yield if each molecule of fatty acid gave rise to 1 molecule of oxalic acid.

The carbon dioxide was determined as usual and found to be 0.668 gm. or 47.0 per cent of that required by Reaction B.

The volatile acids could not be determined satisfactorily owing to their non-volatility, their insolubility in dilute alkali, when it is attempted to determine them by titration, and their condensation as crystals on the walls of condensers.

Presumably results similar to those obtained with the lower fatty acids were obtained in part, but the data do not satisfactorily prove it.

*Oxidation of  $\alpha$ -Hydroxy-n-Stearic Acid*—4.2 gm. of free  $\alpha$ -hydroxy-n-stearic acid were obtained from the barium salt as before. This was taken up in sufficient sodium hydroxide, made up to 400 cc., and treated with 465 cc. of 0.1 M potassium permanganate.

The colorless filtrate contained 0.423 gm. of oxalic acid, which is a 33.6 per cent yield on the basis of the usual assumption.

The carbon dioxide formed was determined in the usual way and found to be 0.445 gm., which is a 36.2 per cent yield on the basis of Reaction B.

#### SUMMARY

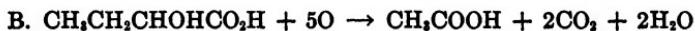
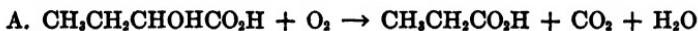
1. Ten of the  $\alpha$ -hydroxy fatty acids with straight chains and 3 or more carbon atoms were synthesized.
2. The sodium salts of these acids were oxidized with potassium permanganate, in the presence of little or no excess alkali.
3. For the lower members of the series the amount of permanganate used was the amount, as determined by test experiments, that could be reduced without undue effort (*i.e.* heating).

For the higher members 5 atoms of oxygen were used for each molecule of fatty acid present.

4. In this work it was found that the rule laid down by Popoff in 1871, on the basis of the oxidative behavior of two  $\alpha$ -hydroxy fatty acids, that  $\alpha$ -hydroxy fatty acids undergo oxidation by the loss of 1 carbon atom, is not true for  $\alpha$ -hydroxybutyric acid.

5. This constitutes a confirmation of the results of Markownikoff (1875), obtained with  $\alpha$ -hydroxybutyric acid, who found that some molecules lost 1 carbon atom while others lost 2 carbon atoms. In other words, two types of oxidative breakdown occurred simultaneously.

6. The results given here indicate that under the conditions used the two types of breakdown occur almost equally. These may be summarized as follows:



7. The results for the series show a shift of the point of rupture of the chain involving a loss of 1 carbon atom from lactic acid with the formation of acetic acid and carbon dioxide, to a loss of 2 carbon atoms from  $\alpha$ -hydroxyheptylic acid with the formation of valeric acid and 2 molecules of carbon dioxide. For the higher fatty acids no evidence was obtained that there is any difficulty about the loss of 2 carbon atoms at a time.

8. The major portion of this shift occurred on passing from the 3-carbon acid to the 5-carbon acid, with  $\alpha$ -hydroxybutyric acid acting as the pivot upon which the change is made. It is not without biological interest and importance that this 4-carbon acid should hold this position.

9. These results indicate that the existing reason for excluding  $\alpha$ -oxidation from consideration for the *in vivo* oxidation of fatty acids is not necessarily valid, and that some of the results visualized as consequent upon an initial  $\beta$ -oxidation also occur following an initial  $\alpha$ -oxidation.  $\alpha$ -Oxidized fatty acids do not necessarily lose but 1 carbon atom as has been believed heretofore.

10. The existence of such a shift in the point of rupture gives a definite experimental basis for searching for others.

11. No evidence was obtained indicating that the mechanism

of the oxidation of  $\alpha$ -hydroxy fatty acids is influenced by whether the carbon chain contains an even or an odd number of carbon atoms.

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## THE OXIDATION OF METABOLITES

### II. THE SHIFT IN THE POINT OF RUPTURE IN A GIVEN $\alpha$ -HYDROXY FATTY ACID IN THE PRESENCE OR ABSENCE OF FREE ALKALI

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Among the unsolved problems relating to the oxidation of fatty acids *in vivo* is the question as to why, if these acids are oxidized in the organism with the loss of 2 carbon atoms at a time, larger amounts of the short chain intermediates are not found in the fluids and tissues of the organism. If the generally accepted views of the mechanism of fatty acid oxidation under biological conditions are correct, then it would be necessary to postulate that the ease of oxidation of fatty acids increases as the chain becomes shorter in order to explain the "clean-up" of short chains as it exists in normal circumstances in the organism. As a matter of fact fatty acids in general have proved to be so resistant to oxidative attack by oxidizing agents *in vitro* that very few chemical data are available for the consideration of this question. Owing to the fact that the alkali salts of the lower fatty acids seem to be usually readily oxidized in the organism, no urgent need for a chemical investigation of the problem has arisen.

In view of the fact that in Paper I it was shown that on passing up the homologous series of  $\alpha$ -hydroxy fatty acids from lactic acid to  $\alpha$ -hydroxycaproic acid there is a gradual shift from a preponderance of a loss of 1 carbon atom from the chain with lactic acid to a preponderance of a loss of 2 carbon atoms from the chain with  $\alpha$ -hydroxycaproic acid, it seemed wise to extend certain fragmentary observations previously reported to see whether the same gradual shift could be produced with a given  $\alpha$ -hydroxy fatty acid.

Previous data available on this subject are not abundant. In 1912 (1) some oxidations of lactic acid reported by Denis (2) were repeated in order to get complete returns on the products of oxidation. The oixdation of sodium lactate with potassium permanganate in the presence of 9 molecules of potassium hydroxide gave results as follows: oxalic acid 6.4 gm., acetic acid 0.142 gm., carbon dioxide 4.40 gm. These results show that lactic acid is oxidized under the conditions used in accordance with three reactions:

1.  $\text{CH}_3\text{CHOHCO}_2\text{H} + 2\text{O} \rightarrow \text{CH}_3\text{CO}_2\text{H} + \text{CO}_2 + \text{H}_2\text{O}$
2.  $\text{CH}_3\text{CHOHCO}_2\text{H} + 5\text{O} \rightarrow (\text{CO}_2\text{H})_2 + \text{CO}_2 + 2\text{H}_2\text{O}$
3.  $\text{CH}_3\text{CHOHCO}_2\text{H} + 6\text{O} \rightarrow 3\text{CO}_2 + 3\text{H}_2\text{O}$

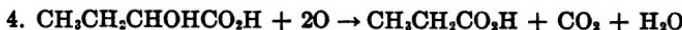
In the results reviewed above 87 per cent of the lactic acid was oxidized according to Reaction 2, 3 per cent according to Reaction 1, and 10 per cent according to Reaction 3.

The results on the oxidation of lactic acid with potassium permanganate in neutral solution, reviewed in Paper I (*cf.* also (3) p. 216), show that in the absence of considerable alkali the oxidation with permanganate involves the same three reactions, but that there is a distinct shift in their importance, for we now have about 83 per cent of the lactic acid oxidized according to Reaction 1, about 3.2 per cent according to Reaction 2, and the remainder according to Reaction 3.

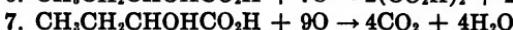
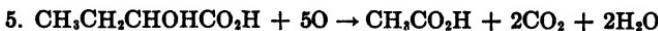
This was interpreted as showing that increasing alkalinity causes a shift in the main point of rupture of the carbon chain from the bond between the carboxyl carbon atom and the  $\alpha$ -carbon atom to that between the  $\alpha$ - and  $\beta$ -carbon atoms. Just how gradually this shift takes place is brought out clearly in earlier work on the oxidation of acetone (4) in which the same labile intermediate, namely pyruvic acid, is involved.

There are also some data available on the oxidation of  $\alpha$ -hydroxybutyric acid ((3) p. 218) which indicate that there is a profound change in the oxidation of sodium  $\alpha$ -hydroxybutyrate upon addition of alkali to the solution before addition of potassium permanganate. When this salt was oxidized in the presence of 4 molecular equivalents of sodium hydroxide, 0.400 gm. of acetic acid, 0.872 gm. of oxalic acid, and 0.83 gm. of carbon dioxide were

formed. No evidence was obtained that propionic acid was formed thus:



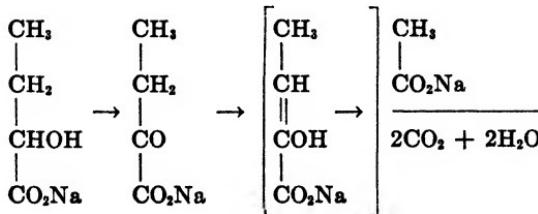
However, the following three reactions did occur:



From the above data it is obvious that about 50 per cent of the salt was oxidized in accordance with Reaction 5, about 37 per cent according to Reaction 6, and the remainder according to Reaction 7.

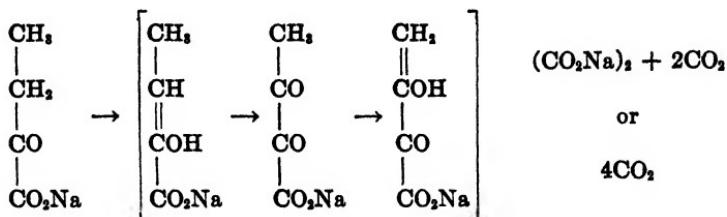
If the data of this preliminary experiment are correct, about 75 per cent of the  $\alpha$ -hydroxybutyric acid used was virtually oxidized to carbon dioxide, owing to the fact that oxalic acid is so readily oxidized to carbon dioxide in slightly acid solutions. The stability of the oxalate in this case is definitely contingent upon the presence of excess alkali.<sup>1</sup>

In the light of all the facts available there are, it appears, but two oxidation mechanisms involved in the result obtained. The first involves the intermediate formation of  $\alpha$ -ketobutyric acid which upon enolization and subsequent oxidation gives the products of Reaction 5.



<sup>1</sup> In all oxidations with potassium permanganate in neutral or alkaline solution any oxalic acid obtained may be regarded as a product of complete oxidation. Its presence is due solely to the fact that there is enough alkali present in the system to convert it into the normal oxalate. The acid oxalate would not survive oxidation. It is therefore possible in such oxidations as these that oxalic acid may be present in appreciable amounts in the earlier stages of the oxidation and that it is actually diminished in amount later on as additional acid products accumulate and neutralize

The other represents more profound changes that are presumably initiated in the same way.



The stages enclosed in the brackets could presumably not be isolated because of their extreme lability.

In this connection it should be noted that we have two groups furnishing hydrogen for the formation of the enol—the  $\text{CH}_2$  and the  $\text{CH}_3$  groups. It is well known that the hydrogen of the methylene group is more labile than that of the methyl group and that consequently the enol will be more readily formed with  $\alpha$ -ketobutyric than with pyruvic acid. This is also supported by the fact ((3) p. 220) that when  $\alpha$ -hydroxybutyric acid is oxidized in a phosphate buffer mixture oxidation apparently takes place entirely according to Reaction 5.<sup>2</sup> Under these conditions the hydrogen of the methylene group is labile, while that of the methyl group is not.

The latter of the two mechanisms above deserves a more detailed investigation because of the fact that it represents an interpreta-

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the available alkali. The presence of oxalic acid is therefore an accident and from the point of view of the work in this paper is regarded as "potential" carbon dioxide, since it would only be necessary to reduce the alkalinity in order to permit of its complete oxidation to carbon dioxide in these systems.

<sup>2</sup> It should be stated here that many data in support of the views expressed and implied in this paper and the preceding one could be cited from the work of Evans and his pupils, much of which is so conveniently summarized in his William H. Nichols Medal Address for 1929 (5). It is the author's opinion that, when all of the facts become available, the oxidative mechanism of fatty acids and of carbohydrates in the living organism will be found to be more closely related than there is at present any basis for believing. The data given in this paper show that the  $\alpha$ -hydroxy fatty acids here investigated really behave much like sugars. In this connection we need only recall that in the living organism lactic acid is potential sugar.

tion of the only known experimental data upon which an explanation of fatty acid oxidation can be constructed that would not involve the formation of more difficultly oxidized normal fatty acids as intermediate products, which in turn would compete on somewhat even terms with the mother substance for available oxygen. Judging from the quantitative composition of the fatty acids recovered from organisms (conditions involving prolonged autolysis or putrefaction being excluded, of course), they consist almost entirely of long chain fatty acids. It is as though we had opened a box of bead necklaces and found that nearly all of the necklaces were intact. When not intact, we found that the necklaces were completely broken up; in other words there would be no short necklaces. By extending the analogy a bit further we see that the oxidation of a fatty acid in the organism appears to resemble the disruption of our necklace, and that any chemical situation in which such a result can be achieved is likely to throw some light upon the process as it occurs in the organism. Of course, the biologist will say that the use of alkali as an activator removes any value that the results might have to the biologist. However, we have only to recall that the activation of glucose by alkali of greater and smaller concentrations was studied for many years and gave an *in vitro* analogue of the behavior of glucose in the normal and diabetic individual, respectively. No one thought that insulin would be a true alkali whenever it should be discovered. Similarly Oparin's work (6) on chlorogenic acid as an oxidative catalyst in plants showed that *in vitro* alkali was required to activate the oxidizing system, but that alkali could be replaced by an extract of the plant and then oxidation took place *in vitro* at H<sup>+</sup> concentrations well below pH 7.00. Other similar instances could be cited. In general, we may say that the use of alkali as the activator is justified by the facts that it is a known substance, that it is conveniently available, that it can be quantitatively controlled, and that its effects resemble in a general way those of the specific activators that function in the organism.

#### EXPERIMENTAL

The methods used were the same as those used in the previous papers. For the sake of clarity and brevity the analytical details are suppressed and only the summarized results are given.

*Oxidation of Lactic Acid in Presence of Variable Amounts of Alkali*—A solution of sodium lactate containing 1 gm. of lactic acid in 200 cc. was prepared from hydrated zinc lactate by decomposing it with the requisite amount of sodium carbonate. Portions of 200 cc. were placed in a series of nine flasks. The amount of sodium hydroxide (2.5 N solution) necessary to constitute the molecular amount, as calculated in Table I, was then

TABLE I  
*Oxidation of  $\alpha$ -Lactic Acid*

1.00 gm. of lactic acid was used in each experiment.

Experiment No.	NaOH added	KMnO <sub>4</sub> added	Oxalic acid		CO <sub>2</sub>		Acetic acid		Per cent lactic acid used recovered
			mols.	gm.	gm.	per cent*	gm.	per cent‡	
1	None	3.16	None		0.724	148.1	0.423	65.1	92.7
2	0.5	3.87	0.487	48.7	0.551	112.7	0.247	37.0	94.7
3	1.0	4.50	0.748	74.8	0.543	111.4	0.124	18.6	99.4
4	1.5	4.18	0.783	78.3	0.546	111.9	0.073	10.9	96.8
5	2.0	4.81	0.842	84.2	0.559	114.5	0.053	7.9	99.5
6	3.0	4.58	0.897	89.7	0.534	109.4	0.025	3.7	98.7
7	4.0	4.42	0.894	89.4	0.551	112.4	?§	?	97.2
8	8.0	4.42	0.965	96.5	0.490	100.4	?	?	97.8
9	16.0	4.42	0.983	98.3	0.494	101.2	?	?	99.2

\* The calculation of percentage yields in this and the following tables is arbitrary. The per cent yield of oxalic acid is calculated on the assumption that each molecule of lactic yields 1 molecule of oxalic acid.

† On the basis that one-third of the carbon present in the lactic acid used is oxidized to CO<sub>2</sub> (calculated yield is then 0.488 gm.).

‡ Calculated on the assumption that each molecule of lactic acid yields 1 molecule of acetic acid.

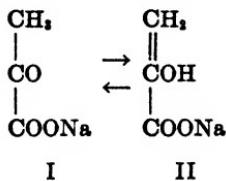
§ In these cases no measurable amount of volatile acids was obtained.

added. The amount of potassium permanganate necessary to oxidize off 1 carbon atom according to the following reaction, CH<sub>3</sub>CHOHCO<sub>2</sub>H + 2O → CH<sub>3</sub>CO<sub>2</sub>H + CO<sub>2</sub> + H<sub>2</sub>O, was then added; i.e., 147 cc. of a 0.1 M solution or 2.33 gm. of potassium permanganate. After this had been completely reduced, small portions (10 or 5 cc.) at a time were added from day to day until the last portion added was reduced only after several days. The flasks were kept at room temperature throughout the oxidation,

although warming would have facilitated the oxidation. This was done mainly so that the series would be comparable. When carried out in this way, the oxidation required several weeks on the average. The less alkaline oxidations were completed sooner than the others, owing to the slow disappearance of the green potassium manganate in the more alkaline ones.

The results are given in Table I. In this case very little comment is necessary, because no novel results are given. The series is convenient, however, because it connects up all of the isolated results previously mentioned in the introduction and shows the gradual transition from one type of oxidation to the other. In the first member of the series 65 per cent of the calculated amount of acetic acid was formed. The amount of carbon dioxide was nearly 50 per cent in excess of that expected on the basis of the reaction given above, although even with this only 92 per cent of the lactic acid was recovered in the form of oxidation products. This suggests that part of the lactic acid is oxidized completely to carbon dioxide, through the intermediate stage of oxalic acid, or perhaps that a small portion of the lactic acid remained unoxidized. In the second member of the series there is already enough alkali present to facilitate this mechanism and to preserve a large part of the oxalic acid formed as normal sodium oxalate. There is still some complete oxidation to carbon dioxide, however, as indicated by the extra carbon dioxide.

In order to visualize the oxidation of lactic acid in these cases it is necessary to think of a dynamic system in which the component forces, and therefore the chemical components, are changing with the increasing alkalinity of the series. Thus we see the yield of acetic acid decreasing in the series because the enolization of the intermediate stage—namely pyruvic acid—increases with increasing alkalinity. In other words, we get less and less acetic acid because the amount of pyruvic acid that exists as such is decreasing, while the amount of it that exists in the iso form is increasing. The change in the products of oxidation is therefore



due to a shift in the equilibrium to the right. We could probably assert that the percentage yield of acetic acid formed determines the average per cent of pyruvic acid molecules that were oxidized while in the normal form (I), and the other products obtained indicate the average per cent of the iso form (II). We say average per cent because the available alkali upon which enolization depends varies throughout the oxidation. It is, in fact, decreasing because all of the products of oxidation are acids.

As was stated above, the appearance of oxalic acid among the end-products is due to the fact that normal sodium oxalate is not oxidized by potassium permanganate, while acid sodium oxalate is oxidized. This acid will therefore survive in proportion as the alkalinity increases in the series but its non-survival in Experiment 1 cannot be regarded as proof of its non-formation as an intermediate. The writer would prefer to leave the question of its actual formation open in this case, since the details of the oxidation of isopyruvic acid are not known at present.

*Oxidation of  $\alpha$ -Hydroxybutyric Acid in Presence of Variable Amounts of Alkali*—The hydrated zinc  $\alpha$ -hydroxybutyrate described in Paper I was decomposed with sodium carbonate and a solution containing 1 gm. of the free acid in 200 cc. was prepared. The requisite amount of alkali was added, as given in Table II. The results of two series of experiments are shown in Table II. An excess of 0.1 M potassium permanganate was measured out for each experiment and portions were added gradually as required after the first 125 cc. had been added at one time and reduced. The oxidation was brought to an end as described in the previous series with lactic acid. In Experiments 12 and 13 a very considerable excess of potassium permanganate was added by mistake so that additional sodium  $\alpha$ -hydroxybutyrate had to be added to reduce the excess permanganate (0.20 gm. in Experiment 12 and 0.30 gm. in Experiment 13). No additional alkali was added. The molecular ratio of alkali as given was the amount present at the beginning of the oxidation.

Series 2 was carried out in the same way and was designed to reveal the stages in the changes of oxidation. In the first two oxidations (Experiments 1 and 2) some acid potassium phosphate was added in order to see to what extent the yield of propionic acid could be increased by this means; i.e., by the addition of a

TABLE II  
*Oxidation of  $\alpha$ -Hydroxybutyric Acid*

1.00 gm. of  $\alpha$ -hydroxybutyric acid as the sodium salt was used in each experiment. Additional amounts were added in Experiments 12 and 13, as explained in the text.

Experiment No.	Series No.	NaOH added	KMnO <sub>4</sub> added	Oxalic acid	Propionic acid	Acetic acid	$\alpha$ -Hydroxybutyric acid recovered as volatile acids		CO <sub>2</sub> on basis of acetic and propionic acids		$\alpha$ -Hydroxybutyric acid recovered		
							mols.	gm.	per cent	gm.	per cent	per cent	
1	2	*	2.33	None	0.436	61.3†	0.157	27.6†	88.8	None	0.588§		
2	2	*	2.49	"	0.410	57.2‡	0.177	30.8‡	88.4	0.294†	0.503	58.4	
3	1	0	3.19	"	0.332	46.7	0.195	33.9	80.6	0.615	0.486	126.5	
4	2	0	2.58	"	0.334	47.0	0.203	35.3	82.3	0.557	0.496	112.3	
5	2	0.5	3.87	0.404	46.6	0.139	19.5	0.311	53.9	73.5	0.529	0.541	97.7
6	1	1.0	4.47	0.724	83.8	0.043	6.0	0.282	48.9	63.6	0.503	0.439	114.5
7	2	1.0	4.50	0.695	80.4	0.066	9.2	0.322	55.8	65.0	0.412	0.511	80.6
8	2	2.0	4.74	0.802	92.6	None	0	0.345	59.9	59.9	0.462	0.507	91.0
9	2	3.0	4.50	0.882	101.8	"	0	0.336	58.3	58.3	0.557	0.491	113.4
10	1	4.0	5.14	0.983	103.4	"	0	0.331	52.3	52.3	0.307	0.482	63.9
11	1	5.0	4.44	1.022	118.0	"	0	0.307	53.4	53.4	0.374	0.448	83.4
12	1	10.0	6.84	1.263	112.4	"	0	0.271	36.2	36.2	0.749	0.396	100
13	1	20.0	9.17	1.484	115.0	"	0	0.270	31.2	31.2	0.709	0.393	100

\* In Experiment 1, 50 cc. of 0.3 M KH<sub>2</sub>PO<sub>4</sub> were substituted; in Experiment 2, 30 cc.

† Much CO<sub>2</sub> was lost owing to the acidity of the solution.

‡ This is the per cent of the calculated amount that was found.

§ Based on volatile acids recovered. The discrepancy in the acid recovered when compared with the acid used may in part be due to complete oxidation to carbon dioxide with the loss of part of it from the solution due to inadequate alkali, and in part to incomplete oxidation.  $\alpha$ -Hydroxybutyric acid remaining unoxidized was not determined because the discrepancy was at first attributed to experimental error and in some degree to the loss of carbon dioxide.

|| This includes the carbon dioxide in excess of that required for the acetic acid found.

very weak acid which would at the same time not interfere with the analytical procedures used.

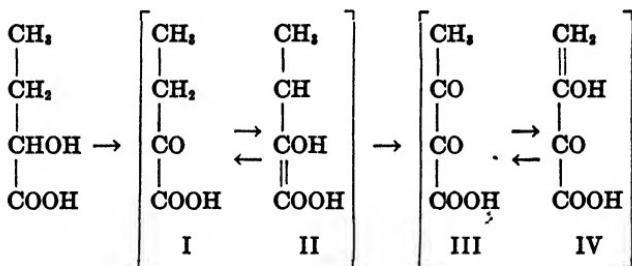
The results given in Table II require little comment. The data report the quantity of oxalic, propionic, and acetic acids, and carbon dioxide actually found. The percentages of oxalic, acetic, and propionic acids are calculated on the basis that each molecule of  $\alpha$ -hydroxybutyric acid yields but 1 molecule of each. So that on this basis it is evident that in Experiment 13 more than 1 molecule of oxalic acid is obtained from each molecule of the fatty acid burned. The per cent of carbon dioxide is calculated on the basis of the acetic and propionic acids found as required by Reactions 4 and 5 given above. In this form the data are somewhat more easily visualized.

In Experiment 1 the propionic acid recovered was 61.3 per cent of that theoretically possible, while about 27.6 per cent of the calculated amount of acetic acid was obtained. Much or all of the carbon dioxide was lost during oxidation in Experiments 1 to 4. As soon as any alkali is added, oxalic acid appears among the products, while propionic acid almost disappears. Acetic acid reaches its maximum when 2 molecules of alkali have been added and then gradually falls with considerable corresponding increases in oxalic acid and carbon dioxide. In Experiment 13 only about 16.0 per cent of the carbon present in the original  $\alpha$ -hydroxybutyric acid persists as a fatty acid, *i.e.* as 0.27 gm. of acetic acid, while the remainder has been potentially converted into carbon dioxide (*i.e.*, recovered as carbon dioxide and oxalic acid). As has been stated before, the oxalic acid persists in these alkaline solutions because the normal salt is stable. In this experiment the oxidation products recovered account for all of the  $\alpha$ -hydroxybutyric acid used.

It is important to note that the results in Table II prove definitely that in the formation of oxalic acid 2 carbon atoms have been detached from the chain in one piece, unless the reader is ready to insist that individual carbon atoms are detached and then united to form oxalic acid. If the data allow of the former interpretation, we have here actual evidence that an  $\alpha$ -hydroxy fatty acid undergoes oxidation with the loss of 2 carbon atoms at a time and in one piece. In the case of lactic acid we could not prove this point because the other product of oxidation was carbon

dioxide and we could not declare positively from which end of the carbon chain it arose. Here, because of the simultaneous formation of acetic acid, it is possible to identify the original parts of the molecule in the fragments.

All of the results obtained are comprehensible if the existence of the following intermediate products and equilibrium forms is granted.



Formula (I) by further oxidation gives rise to propionic acid and carbon dioxide. (II) and (III) by further oxidation give rise to acetic and oxalic acids or acetic acid and 2 molecules of carbon dioxide depending on circumstances. (IV) by further oxidation gives rise to 2 molecules of oxalic acid, or 1 molecule of oxalic acid and 2 molecules of  $\text{CO}_2$ , or 4 molecules of  $\text{CO}_2$ , depending on circumstances. Brackets are used to represent hypothetical stages or hypothetical substances in some cases, which it may not be possible to prepare because of instability.

*Oxidation of  $\alpha$ -Hydroxyvaleric Acid and of  $\alpha$ -Hydroxycaproic Acid in Presence of Variable Amounts of Alkali*—The zinc salts of these two acids, described in Paper I, were decomposed with sodium carbonate and solutions containing 1 gm. of the free acid as the sodium salt in 200 cc. were prepared. These zinc salts are wet by water with difficulty. The decomposition was most readily carried out by grinding them in a mortar with the requisite amount of sodium carbonate and some water. After decomposition was complete, the mass was diluted, warmed, and zinc hydroxide filtered off in the usual manner.

The filtrate containing some excess sodium carbonate was then treated with a slight excess of phosphoric acid and boiled to remove carbon dioxide. The solution was then neutralized as nearly as possible to litmus paper. This solution contained some sodium acid phosphate and was therefore buffered in such a way

as to give in the process of oxidation a rather large proportion of the fatty acid with 1 carbon atom less than the acid subjected to oxidation.

TABLE III  
*Oxidation of  $\alpha$ -Hydroxyvaleric Acid*

1.00 gm. of  $\alpha$ -hydroxybutyric acid was used in each experiment.

Experiment No.	NaOH added	Oxalic acid			Butyric acid	Propionic acid	$\alpha$ -Hydroxyvaleric acid recovered as volatile acids	CO <sub>2</sub>		Approximate per cent $\alpha$ -hydroxyvaleric acid recovered as oxidation products
		mols.	gm.	per cent				per cent	gm.	
1	None	None	0	76.14	20.8	96.8	0.535	143.8		98
2	0.5	0.5014	65.7	?	87.2	87.2	0.314	84.4		95
3	1.0	0.6953	91.2	Trace	Largely	82.0	0.254	68.3		99
4	2.0	0.7756	101.7	?	Mostly	62.9	0.280	75.3		92
5	6.0	0.8816	115.6		Much acetic acid	53.3	0.267	71.8		93

\* On the basis of 1 carbon atom.

TABLE IV  
*Oxidation of  $\alpha$ -Hydroxycaproic Acid*

1.00 gm. of  $\alpha$ -hydroxycaproic acid was used in each experiment.

Experiment No.	NaOH added	Oxalic acid			Valeric acid	Butyric acid	$\alpha$ -Hydroxycaproic acid recovered as volatile acids	CO <sub>2</sub>		Approximate per cent $\alpha$ -hydroxycaproic acid recovered as oxidation products
		mols.	gm.	per cent				per cent	gm.	
1	None	None	0	62.7	37.3	96.3	0.468	140.5		96
2	0.5	0.3508	51.4	12.6	84.4	90.8	0.328	98.5		96
3	1.0	0.6199	90.9	?	—100	88.8	0.200	66.6		100
4	2.0	0.7242	106.2		Largely	76.0	0.266	79.8		99
5	6.0	0.7689	112.7		Mostly	55.3	0.287	86.2		89

As before, the requisite amount of alkali as indicated in Tables III and IV was added and then potassium permanganate of known concentration was added until oxidation was complete. The results are given in Tables III and IV.

It is not necessary to discuss the results obtained. As was expected, analysis in these cases is complicated by the fact that more than 2 carbon atoms are lost from the fatty acid chains and so it was impossible to describe adequately the volatile acids obtained when as much as 1 molecule of extra alkali or more was present in the solution at the beginning. This was to be expected on the basis of the results reported in the previous paper.

In order to see whether the volatile acid products were constituted as stated, the volatile acid from Experiment 1 of the  $\alpha$ -hydroxyvaleric acid series (Table III) was subjected to a more

TABLE V  
*Fractionation of Unknown Mixture*

	Fraction 1	Fraction 2	Residual fraction
1st half, 120 cc.....	14.07 cc.	9.06 cc.	4.16 cc.
2nd " 120 " .....	5.69 "	4.06 "	2.42 "
	19.76 cc.	13.12 cc.	6.58 cc.
Acid in 1st half.....	71.2%	69.05%	63.2%
Butyric acid.....	19.14 cc.	10.54 cc.	2.26 cc.
Propionic " .....	0.62 "	2.58 "	4.52 "
Total butyric acid.....	31.94 cc. or 80.95%		
" propionic " .....	7.52 " " 19.05%		

The volatility factor used in the calculations was 58.8 per cent for propionic and 71.6 per cent for butyric acid.

rigid fractionation, similar to that used with the products of  $\alpha$ -hydroxybutyric acid oxidation in Paper I.

The volatile acids from one-half of the oxidation mixture were recovered in the usual way by distillation from the aqueous solution acidified with phosphoric acid. This was diluted to 600 cc. and two 200 cc. fractions were distilled off. The two fractions and the residue were now diluted to 240 cc. and 120 cc. were distilled off from each of them. All of these were then titrated with 0.1 N sodium hydroxide. The results obtained are shown in Table V.

It hardly seemed worth while to set up a synthetic mixture having this composition, because it was thought that there was probably enough acetic acid present to distort the results. Never-

theless, it was done. The fractionation was carried out exactly as above and the results are given in Table VI.

It is interesting to note that the main deviation occurs in the last fraction. If the first 120 cc. of this fraction had required 4.00 cc. of alkali instead of 3.89 cc., the final figures would have been 80.27 per cent for butyric acid and 19.73 per cent for propionic acid. It goes without saying that this deviation is within the experimental error and so the results not only reveal the essential purity of the products as reported but also the range of the experimental error when applied to acids of this sort.

TABLE VI  
*Fractionation of Synthetic Mixture*

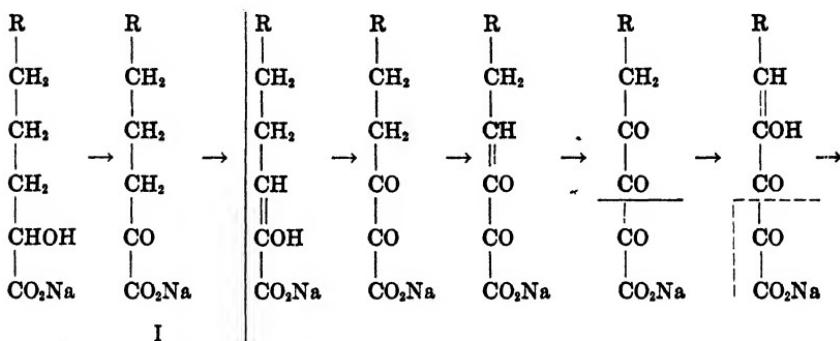
	Fraction 1	Fraction 2	Residual fraction
1st half, 120 cc.....	13.60 cc.	8.67 cc.	3.89 cc.
2nd " 120 " .....	5.48 "	3.93 "	2.48 "
	19.08 cc.	12.60 cc.	6.37 cc.
Acid in 1st half.....	71.2%	68.80%	61.06%
Butyric acid.....	18.59 cc.	9.92 cc.	1.17 cc.
Propionic " .....	0.49 "	2.68 "	5.20 "
Total butyric acid.....	29.68 cc. or 77.99%		
" propionic " .....	8.37 " " 22.01%		

#### DISCUSSION

The purpose of this paper was twofold. (1) It was desired to learn whether the point of rupture of  $\alpha$ -hydroxy fatty acids could be shifted from a preponderance of a loss of 1 carbon atom from the fatty acid molecule to the loss of 2 carbon atoms by changing the hydroxyl ion concentration of the solution. (2) Additional information on the tendency of these  $\alpha$ -hydroxy acids to undergo further oxidation was desired.

The results as far as the first purpose is concerned were quite definite and it was found that the point of rupture is sensitively influenced by the alkali concentration of the solution. The transition from the loss of 1 carbon atom to the loss of 2 was practically complete when as much as 1 extra molecule of alkali had been added. Under these conditions a considerable amount of oxalic acid was formed which indicates that, to a considerable extent at

least, the 2 carbon atoms are detached in one piece and that this then gives rise to oxalic acid. At the same time there is a definite tendency for other products, involving additional shortening of the carbon chain, to be formed, in case the acid used has 4 or more carbon atoms in each molecule. This is discussed for each acid individually and a general schematic interpretation may be given as follows:



$\begin{array}{c} \text{CO} \\ | \\ \text{CO}_2\text{Na} \end{array}$        $\text{CO}_2\text{Na}$ , or  $(\text{CO}_2\text{Na})_2$ , or 2Na carbonate

$\begin{array}{c} \text{CO} \\ | \\ \text{CO} \\ | \\ \text{CO}_2\text{Na} \end{array}$       The remaining 4 carbon atoms as Na carbonate or  $(\text{CO}_2\text{Na})_2$

The oxidation of the  $\alpha$ -keto acid (I) gives rise to the fatty acid having 1 less carbon atom. The enolic and keto compounds within the brackets give rise to the loss of 2 carbon atoms at once or multiples of two may be lost successively two at a time.

This scheme readily explains the appearance of short chain acids where longer ones alone were expected. Moreover, it enables one to visualize the oxidation of a long chain fatty acid without the formation of saturated fatty acids as intermediate products. If this scheme represents the facts even approximately, the oxidation of a fatty acid is like the burning of a fuse, and constitutes a chemical example of the "all or none" principle of physiology. It is proposed to work this out more fully under other conditions,

under the conviction that the effect should be more fully and effectively realizable when the proper conditions are found.

Having found in Paper I that there is a shift from the loss of 1 carbon atom to the loss of 2 upon lengthening the carbon chain in the fatty acid, and having found in this paper that a similar result is obtained with a given  $\alpha$ -hydroxy fatty acid by increasing the alkalinity of the solution, it would not be surprising if other ways of bringing about this change may be found.

#### SUMMARY

1. The sodium salts of four of the  $\alpha$ -hydroxy fatty acids with straight chains were oxidized with potassium permanganate in the presence of variable amounts of alkali.
2. The results show a shift of the point of rupture of the chain from a loss of 1 carbon atom largely, to a loss of 2 carbon atoms largely, as soon as there was 1 molecule of alkali in excess.
3. This result was obtained with lactic,  $\alpha$ -hydroxybutyric,  $\alpha$ -hydroxyvaleric, and  $\alpha$ -hydroxycaproic acids.
4. When the 2 carbon atoms were lost from the chain, a considerable portion of this could be identified as oxalic acid. This indicates that in large part the 2 carbon atoms were severed in one piece.
5. The data and available information indicate that the loss of 1 carbon atom is due to the oxidation of the  $\alpha$ -keto acid, while the loss of 2 carbon atoms follows the oxidation of its enol isomer.
6. With more alkali the tendency to complete oxidation, *i.e.* the formation of  $\text{CO}_2$  and oxalic acid alone, is greatly increased. This in turn is interpreted as the result of further enolizations.
7. Since two ways of inducing the shift in the point of rupture of fatty acids have been found, it is suggested that others probably also exist.

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## THE AMINO ACID DEFICIENCIES OF BEEF, WHEAT, CORN, OATS, AND SOY BEANS FOR GROWTH IN THE WHITE RAT

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Only a small amount of work has been done upon the amino acid deficiencies of the protein mixtures occurring in natural foods. Nevertheless, such information should be of practical value in indicating which combinations of foods will be the most advantageous from the standpoint of protein nutrition. If foods containing different amino acid deficiencies are fed together, a protein-supplementing effect of some extent will always result, in the sense that the biological value of the mixture will be greater than the weighted average of the biological values of the component foods. On the other hand, if foods containing the same amino acid deficiencies are fed together, it is obviously impossible for either food to improve the protein value of the other.

In previous publications from this laboratory it has been shown that garden peas (both fresh and canned), potatoes, and whole milk are deficient in cystine (1), the last result being a confirmation of previous experimental work published by Sherman and Merrill (2). The investigations now to be reported were concerned with the amino acid deficiencies of beef, wheat, corn, oats, and soy beans.

Some preliminary experiments on beef, with the paired feeding method (1), had not indicated clearly that the growth-promoting properties of this meat were improved by the addition of cystine. However, the significance of this tentative conclusion was impaired by the fact that only four pairs of rats were used, and partic-

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ularly by the fact that the food intakes of the pair mates were only approximately equated. As it was, three of the four pairs showed better growth for the rat receiving the cystine-supplemented ration in spite of a greater intake of food by the control rat. The greater growth of the control rat in the fourth pair was associated with a greater intake of food. Because of the uncertain significance of these preliminary experiments on the possibility of a cystine deficiency in beef, they were repeated on eight pairs of rats in the present experiment.

No definite information was available on the amino acid deficiency of wheat, though a lysine deficiency was suspected because of the fact that gliadin contains this amino acid in low concentration.

Hogan (3) has concluded that the proteins of corn are deficient in tryptophane and that, when tryptophane is supplied, a lysine deficiency is manifested. However, his rat feeding tests were made not upon the whole protein mixture of corn, but upon a concentrate prepared from ground corn by successive treatments with boiling water acidified with acetic acid, the soluble material being discarded after each treatment. No information was obtained concerning the losses of nitrogen in the discarded portions, so that no conclusion can be drawn as to what fraction of the total proteins of corn remained in the protein concentrate. Whether the conclusions of Hogan will apply to the total protein mixture of corn cannot, therefore, be decided. Hence the relation of both lysine and tryptophane to the nutritive properties of corn proteins was reinvestigated, whole corn being used.

There is no experimental evidence available on the amino acid deficiencies of oat proteins or of soy bean proteins, though in the former case lysine might be suspected and in the latter case cystine. These suspicions were, therefore, investigated in the experiments to be reported below.

#### *Methods*

The meat preparation used was made from round steak of beef. The visible fat was cut away and discarded, and the lean meat remaining was ground in a meat chopper and dried at a low temperature. It was then ground finely, extracted with ether, and dried. As thus prepared it contained 14.85 per cent of nitrogen.

The grains used had the composition shown in Table I. The wheat, corn, and soy beans were obtained from the Agronomy

TABLE I  
*Percentage Composition of Grains Used*

	Water	Crude protein	Ether extract	Ash	Crude fiber	N-free extract
Wheat.....	2.64	12.94	2.09	1.84	2.74	67.75
Soy beans.....	8.63	34.19	20.66	4.11	16.21	16.20
Rolled oats.....	1.48	17.12	6.00	1.68	1.71	72.01
Corn.....	9.81	10.06	5.48	1.26	2.52	70.87

TABLE II  
*Percentage Composition of Experimental Rations*

Constituents	Meat protein		Wheat protein		Oat protein, 8 per cent	Corn protein, 8 per cent	Soy beans protein, 10 per cent
	8 per cent	20 per cent	8 per cent	10 per cent			
Dried, ether-extracted beef.....	9.01	21.5	0	0	0	0	0
Whole wheat.....	0	0	62	77	0	0	0
Rolled oats.....	0	0	0	0	46.7	0	0
Ether-extracted soy beans.....	0	0	0	0	0	0	24
Yellow corn.....	0	0	0	0	0	79.5	0
Salt mixture*.....	4	4	4	4	4	4	4
Butter fat.....	10	10	8	8	8	8	8
Sodium chloride.....	1	1	1	1	1	1	1
Dried yeast.....	1	1	2	2	2	2	2
Cod liver oil.....	1	1	2	2	2	2	2
Starch.....	59.99	47.5	21	6	26.3	0	49
Cellu Flour†.....	4	4	0	0	0	0	0
Sucrose.....	10	10	0	0	10	3.5	10
Total.....	100	100	100	100	100	100	100

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

† The Cellu Flour is a product put out by the Chicago Dietetic Supply House. By actual analysis it contains 38 per cent of crude fiber and only 0.015 per cent of nitrogen.

Department and were of excellent grade. The oats were a standard brand of rolled oats. All grains were finely ground, and, previous to use, the soy beans were extracted with ether and dried;

the final product contained only 4.17 per cent of fat, but the protein content was raised from 34.19 to 41.59 per cent.

The percentage composition of the unsupplemented rations is given in Table II. At each mixing of a ration, the completed mixture was divided into two equal parts. One-half was put into air-tight jars immediately. Into the other half was mixed a small amount<sup>1</sup> of the amino acid whose possible supplementing effect it was desired to measure. These rations were designed in most cases to contain approximately 8 or 10 per cent of protein.

The cystine used in these experiments was obtained from the Eastman Kodak Company, the lysine from the Chemistry Department of the University of Illinois through the courtesy of Dr. C. S. Marvel, and the tryptophane from the Hoffmann-LaRoche, Inc. The purity of all of the amino acids was checked by nitrogen determinations.

The paired feeding method was used throughout these studies as in the preceding studies, eight pairs of rats being used in each comparison of an unsupplemented and a supplemented ration. The two rats of each pair received the same amount of food, the unsupplemented ration in one case and the supplemented ration in the other. With each pair the object of the feeding was always to give as much as would be cleaned up by both rats, and continuous records were kept of all refusals of food necessitating a reduction in the amount weighed out to both members of the pair. In this way it was possible to obtain evidence on the comparative avidity with which supplemented and unsupplemented rations were consumed. The correlation of this evidence with that of the comparative growth-promoting values of the rations is thus possible. In general, except for pairs reduced to a very low level of food consumption, no increases in food offered were made on the day preceding the termination of an experimental week, so as to prevent as far as possible refusals of food on the following day.

<sup>1</sup> Cystine was used always in a concentration of 0.25 per cent of the ration. The lysine deficiencies of wheat and oats and the primary lysine deficiency of corn was tested with a 0.25 per cent concentration of the amino acid. On the other hand, only half this concentration of tryptophane was used as a supplement to the corn rations, while in testing the secondary amino acid deficiency of this cereal, the basal ration contained 0.15 per cent of lysine.

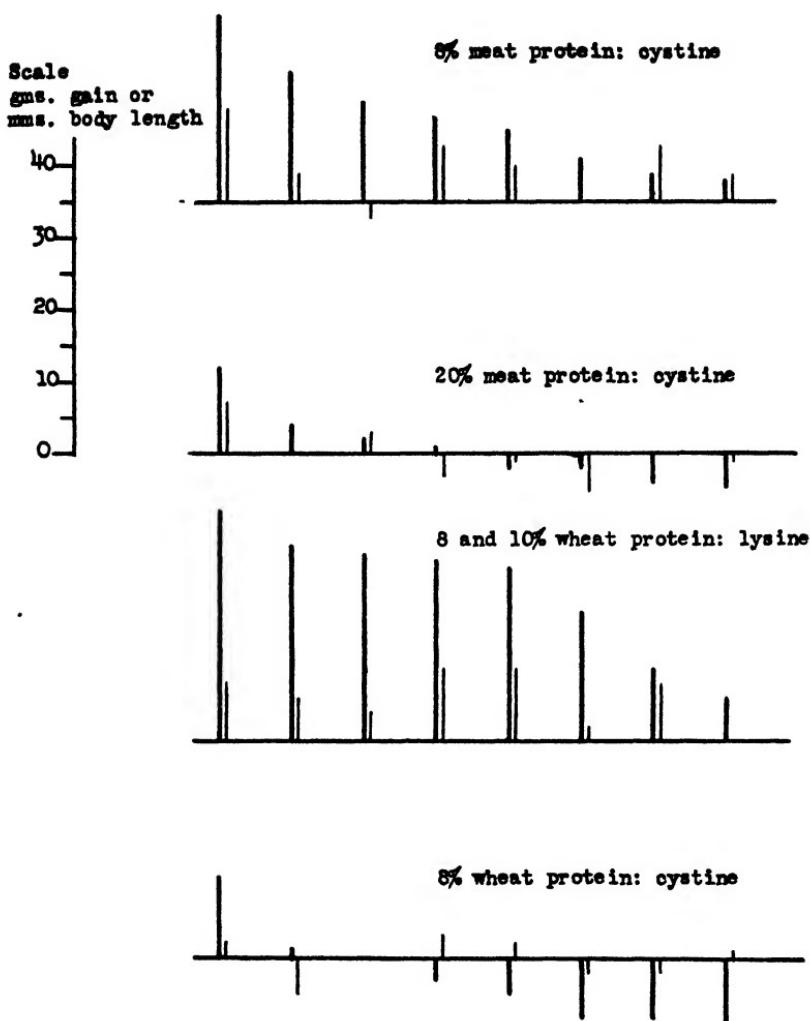


CHART 1. The effects of supplementing rations containing meat and wheat proteins with either cystine or lysine. The vertical lines represent differences between pair mates in total gain (heavy lines) or body length (light lines). If erected above the horizontal base-line, the difference favored the pair mate receiving the amino acid supplement. If erected below the base-line, the difference favored the pair mate receiving the unsupplemented ration. The length of the vertical lines measures the size of the difference between pair mates in accordance with the scale given.

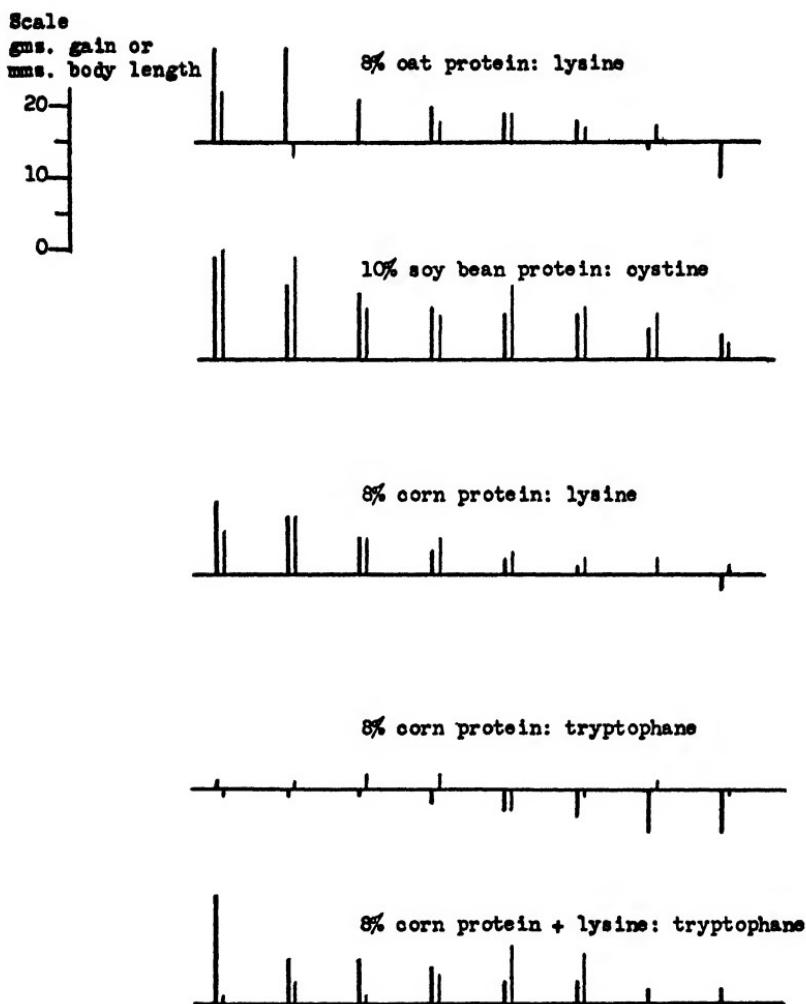


CHART 2. The effects of supplementing rations containing oat, corn, and soy bean proteins with either cystine, lysine, or tryptophane. The vertical lines represent differences between pair mates in total gain (heavy lines) or body length (light lines). If erected above the horizontal base-line, the difference favored the pair mate receiving the amino acid supplement. If erected below the base-line, the difference favored the pair mate receiving the unsupplemented ration. The length of the vertical lines measures the size of the difference between pair mates in accordance with the scale given.

Otherwise the significance of a comparison of weekly gains would be impaired.

The rats were started on experiment at weights of 40 to 60 gm. They were paired according to sex, litter, and initial weight. Body weights were taken weekly and the initial and final weights used were averages of weights taken on 3 consecutive days. The periods of experimental feeding lasted for from 5 to 12 weeks, depending upon the rapidity of gains, generally. At the termination of each experiment the rats were killed by ether and their body lengths from nose to anus determined. The purpose of this measurement was to afford evidence of growth independent of gain in body weight.

#### EXPERIMENTAL

The ultimate results in these paired feeding experiments upon which the conclusions must be based are the differences between pair mates in total gain and in body length. The size of these differences and their consistency determine the probability of the conclusions that they favor. These ultimate results are shown graphically in Charts 1 and 2, while the statistical calculations are given numerically in Table III. The statistical calculations have been made in accordance with the method of "Student" (4) for the interpretation of paired experimental data. The probability computed in each case is determined by the ratio of the mean difference between pair mates to the standard deviation of differences ( $z$ ) and by the number of paired observations ( $N$ ). The value  $P$ , expressed on a scale of 1 for absolute certainty, measures the probability that a mean difference between pair mates as large as that observed could have resulted from chance only. Obviously the smaller  $P$  becomes the more certain is it that the result is not a fortuitous outcome, but is traceable to the one difference in treatment between pair mates, namely the presence of the supplementing amino acid in the ration. If  $P$  is as small as 0.02, it may be concluded that chance has, for all practical purposes, been ruled out as a determinant in the outcome, since in this case chance alone would be expected to produce a divergence between pair mates as great as this only once in 50 trials. In fact, a value of  $P < 0.03$  is according to current biometrical practice a criterion of high significance. In Table III the signs preceding the mean

TABLE III  
Statistical Results of Paired Feeding Experiments with Rats

	Cystine		Lysine		Tryptophane	
	Meat protein 8 per cent	Wheat protein, 8 per cent	Soy bean protein, 10 per cent	Oat protein, 8 per cent	Corn protein, 8 per cent	Corn protein + lysine, 8 per cent
Total gain in weight, gm.						
Mean difference, $M$ .....	+11.6	+0.75	-2.9	+7.4	+4.9	+21.0
Standard deviation, $s$ .....	7.2	5.0	6.5	3.2	5.8	8.4
Ratio, $M:s$ .....	1.6	0.15	0.45	2.3	0.84	0.91
Probability, $P$ .....	0.0019	0.35	0.14	0.0002	0.031	0.024
Body length, mm.						
Mean difference, $M$ .....	+5.0	0.0	+1.1	+8.4	+2.0	+4.0
Standard deviation, $s$ .....	4.4	2.2	2.2	3.8	2.6	2.2
Ratio, $M:s$ .....	1.1	0.50	0.50	2.2	0.77	1.8
Probability, $P$ .....	0.010		0.11	0.0003	0.041	0.001
Comparisons of weekly gains in weight						
No. favoring supplemented ration.....	41	14	21	27	42	29
“ “ unsupplemented ration.....	13	19	35	3	23	25
“ “ neither ration.....	10	7	0	2	7	2
Total refusals of feed by rats on						
Supplemented ration.....	44	11	73	20	56	88
Unsupplemented ration.....	37	25	35	54	108	35

TABLE IV  
*Supplementing Effect of Cystine on Rations Containing Meat Protein  
 Fed to Rats*

The ration containing 8 per cent meat protein was fed 56 days; that containing 20 per cent, 38 days.

	8 per cent meat protein								Control		Cystine					
	Control		Cystine		Control		Cystine		Control		Cystine					
	Pair 1 ♂		Pair 2 ♂		Pair 3 ♂		Pair 4 ♂		Pair 5 ♀		Pair 6 ♀		Pair 7 ♂		Pair 8 ♀	
Initial weight.....	45	45	36	34	45	43	41	42								
Final    ".....	137	155	149	157	172	184	165	192								
Total gain.....	92	110	113	123	127	141	124	150								
Body length.....	18.9	19.3	19.2	19.7	20.4	20.2	19.7	21.0								
Total food.....	381	384	425	425	536	536	554	554								
Initial weight.....	41	42	37	39	36	40	38	34								
Final    ".....	136	141	135	140	142	158	128	130								
Total gain.....	95	99	98	101	106	118	90	96								
Body length.....	18.5	19.3	18.4	18.8	18.7	19.5	18.0	18.0								
Total food.....	480	478	490	490	480	480	468	464								
20 per cent meat protein																
	Pair 1 ♂		Pair 2 ♂		Pair 3 ♂		Pair 4 ♀		Pair 5 ♂		Pair 6 ♂		Pair 7 ♀		Pair 8 ♀	
Initial weight.....	44	44	38	38	40	40	38	38								
Final    ".....	157	155	170	174	165	161	134	146								
Total gain.....	113	111	132	136	125	121	96	108								
Body length.....	19.2	19.1	19.9	19.9	19.9	19.9	18.3	19.0								
Total food.....	332	332	343	343	327	327	329	331								
	Pair 5 ♂		Pair 6 ♂		Pair 7 ♀		Pair 8 ♀		Pair 5 ♂		Pair 6 ♂		Pair 7 ♀		Pair 8 ♀	
Initial weight.....	41	40	40	40	36	37	39	39								
Final    ".....	152	146	144	142	117	119	119	121								
Total gain.....	111	106	104	102	81	82	80	82								
Body length.....	19.1	19.0	19.1	18.6	17.8	17.5	17.7	18.0								
Total food.....	326	326	321	321	294	294	290	292								

TABLE V  
*Supplementing Effect of Cystine on a Ration Containing 8 Per Cent of Wheat Protein Fed to Rats*

The ration was fed 51 days to Pairs 1 to 4, 47 days to Pairs 5 and 6, and 49 days to Pairs 7 and 8.

	Control		Cystine		Control		Cystine		Control		Cystine	
	Pair 1 ♂		Pair 2 ♂		Pair 3 ♀		Pair 4 ♂		Pair 5 ♂		Pair 6 ♂	
Initial weight.....	68	70	62	63	58	61	65	68				
Final     ".....	128	122	133	126	132	124	135	138				
Total gain.....	60	52	71	63	74	63	70	70				
Body length.....	18.4	18.2	18.2	18.0	18.2	18.3	18.7	18.7				
Total food.....	372	372	408	408	425	425	435	435				
	Pair 1 ♂		Pair 6 ♂		Pair 7 ♀		Pair 8 ♀					
Initial weight.....	63	65	68	68	66	67	70	75				
Final     ".....	130	129	140	151	131	127	142	148				
Total gain.....	67	64	72	83	65	60	72	73				
Body length.....	18.5	18.8	18.7	18.9	18.3	18.5	18.6	19.1				
Total food.....	426	426	470	470	441	441	485	485				

TABLE VI  
*Supplementing Effect of Lysine on Rations Containing 8 or 10 Per Cent of Wheat Protein Fed to Rats*

The ration was fed 85 days to Pairs 1 to 5; 62 days to Pairs 6 to 8.

	Control		Lysine		Control		Lysine		Control		Lysine	
	Pair 1 ♂		Pair 2 ♂		Pair 3 ♀		Pair 4 ♂		Pair 5 ♂*		Pair 6 ♂	
Initial weight.....	50	50	51	53	46	47	48	49				
Final     ".....	126	153	133	159	114	125	135	168				
Total gain.....	76	103	82	106	68	78	87	119				
Body length.....	18.6	19.2	18.4	19.4	17.4	18.2	18.6	19.4				
Total food.....	584	584	628	628	536	536	654	654				
	Pair 5 ♂*		Pair 6 ♂		Pair 7 ♀		Pair 8 ♂					
Initial weight.....	48	44	46	46	42	43	39	44				
Final     ".....	126	128	129	155	128	147	128	158				
Total gain.....	78	84	83	109	86	104	89	114				
Body length.....	18.4	18.4	18.7	19.1	18.5	18.7	18.3	19.3				
Total food.....	580	580	519	519	520	520	475	475				

\* The control rat of Pair 5 was a male; the lysine-fed rat a female.

TABLE VII

*Supplementing Effect of Lysine on a Ration Containing 8 Per Cent of Oat Protein Fed to Rats*

The ration was fed 63 days.

	Control		Lysine		Control		Lysine		Control		Lysine	
	Pair 1 ♀		Pair 2 ♂		Pair 3 ♀		Pair 4 ♀		Pair 5 ♀		Pair 6 ♂	
Initial weight.....	46	46	49	50	42	47	49	50				
Final     ".....	122	126	137	151	112	116	126	131				
Total gain.....	76	80	88	101	70	69	77	81				
Body length.....	18.4	18.8	18.6	19.3	17.8	18.0	18.6	18.8				
Total food.....	377	377	407	407	339	339	403	402				
	Pair 7 ♀		Pair 8 ♂									
Initial weight.....	49	49	54	55	51	51	58	59				
Final     ".....	144	139	136	142	123	136	152	159				
Total gain.....	95	90	82	87	72	85	94	100				
Body length.....	18.9	18.9	19.1	19.4	18.6	18.4	19.6	19.6				
Total food.....	425	426	436	436	402	402	465	465				

TABLE VIII

*Supplementing Effect of Cystine on a Ration Containing 10 Per Cent of Soy Bean Protein Fed to Rats*

The ration was fed 35 days.

	Control		Cystine		Control		Cystine		Control		Cystine	
	Pair 1 ♂		Pair 2 ♂		Pair 3 ♂		Pair 4 ♀		Pair 5 ♂		Pair 6 ♂	
Initial weight.....	43	43	60	64	48	48	50	48				
Final     ".....	50	53	60	71	57	61	56	60				
Total gain.....	7	10	0	7	9	13	6	12				
Body length.....	14.1	14.3	14.8	15.4	14.5	15.1	14.3	15.0				
Total food.....	101	101	123	123	107	107	107	107				
	Pair 7 ♀		Pair 8 ♀									
Initial weight.....	44	45	48	48	39	41	44	44				
Final     ".....	40	47	48	58	42	58	38	47				
Total gain.....	-4	2	0	10	3	17	-6	3				
Body length.....	13.5	14.5	14.0	15.4	13.5	15.0	13.4	14.1				
Total food.....	85	85	101	101	111	112	89	89				

TABLE IX  
*Supplementing Effect of Lysine on a Ration Containing 8 Per Cent of  
 Corn Protein Fed to Rats*

The ration was fed 49 days.

	Control		Lysine		Control		Lysine		Control		Lysine	
	Pair 1 ♂		Pair 2 ♀		Pair 3 ♂		Pair 4 ♀		Pair 5 ♀		Pair 6 ♂	
Initial weight.....	45	44	41	41	41	41	43	43				
Final     ".....	76	75	75	83	65	66	77	80				
Total gain.....	31	31	34	42	24	25	34	37				
Body length.....	16.0	16.2	15.9	16.7	15.5	15.7	15.8	16.3				
Total food.....	227	227	247	247	193	193	248	248				
	Pair 7 ♂		Pair 8 ♀		Pair 9 ♂		Pair 10 ♀		Pair 11 ♂		Pair 12 ♀	
Initial weight.....	41	41	43	45	40	41	57	57				
Final     ".....	62	72	63	70	58	61	92	90				
Total gain.....	21	31	20	25	18	20	35	33				
Body length.....	15.1	15.7	15.5	16.0	15.1	15.4	16.4	16.5				
Total food.....	199	199	192	192	165	164	242	242				

TABLE X  
*Supplementing Effect of Tryptophane on a Ration Containing 8 Per Cent of  
 Corn Protein Fed to Rats*

The ration was fed 50 days.

	Control		Trypto- phane		Control		Trypto- phane		Control		Trypto- phane	
	Pair 1 ♀		Pair 2 ♂		Pair 3 ♂		Pair 4 ♂		Pair 5 ♀		Pair 6 ♀	
Initial weight.....	51	53	52	55	51	51	48	50				
Final     ".....	82	78	83	84	104	101	85	81				
Total gain.....	31	25	31	29	53	50	37	31				
Body length.....	16.1	16.0	16.5	16.7	17.3	17.0	16.2	16.3				
Total food.....	282	282	268	268	334	332	264	262				
	Pair 7 ♂		Pair 8 ♀		Pair 9 ♂		Pair 10 ♀		Pair 11 ♂		Pair 12 ♀	
Initial weight.....	53	54	53	53	53	55	51	52				
Final     ".....	103	103	81	80	90	93	91	88				
Total gain.....	50	49	28	27	37	38	40	36				
Body length.....	16.7	16.9	16.2	16.3	17.2	17.1	16.9	16.8				
Total food.....	360	360	292	292	321	323	362	362				

differences between pair mates indicate whether the mean difference favors the supplemented ration (+), or whether it favors the unsupplemented ration (-).

If the average difference between pair mates in any one comparison is statistically significant as determined by this method, it would seem that in all probability the added amino acid has supplemented the protein mixture of the basal ration, increasing its growth-promoting value. It is realized, however, that all other

TABLE XI  
*Supplementing Effect of Tryptophane on a Ration Containing 8 Per Cent Corn Plus Lysine Fed to Rats*

The ration was fed 50 days.

	Control		Trypto- phane		Control		Trypto- phane		Control		Trypto- phane	
	Pair 1 ♀		Pair 2 ♂		Pair 3 ♀		Pair 4 ♂		Pair 5 ♂		Pair 6 ♂	
Initial weight.....	46	49	43	44	41	44	40	41				
Final     ".....	102	111	76	83	69	77	66	70				
Total gain.....	56	62	33	39	28	33	26	29				
Body length.....	16.5	16.8	16.2	16.3	15.3	15.7	14.9	15.7				
Total food.....	317	317	247	250	224	224	226	226				
	Pair 5 ♂		Pair 6 ♂		Pair 7 ♀		Pair 8 ♀					
Initial weight.....	43	44	47	49	45	45	44	46				
Final     ".....	80	83	73	78	96	98	87	104				
Total gain.....	37	39	26	29	51	53	43	58				
Body length.....	16.2	16.1	15.3	16.0	16.3	16.2	16.2	16.3				
Total food.....	235	235	230	230	286	286	311	311				

possible interpretations have not been disposed of (5), though they would seem to be extremely improbable.

At the bottom of Table III, two other types of information are summarized; i.e., a comparison of weekly gains, and a comparison of total refusals of feed. The former constitutes confirmatory evidence of the differential effect upon growth of the two rations being compared, less significant than the comparison of total gain to the extent that food residues at the end of the weekly periods may have exerted an appreciable effect upon the gains

made by pair mates. The latter constitutes interesting evidence of the relation between appetite and dietary balance.

The complete data from which the differences in pair mates have been computed are summarized in Tables IV to XI, inclusive.

The results with the individual foods may be summarized as follows:

*Beef*—The dried lean from beef round was tested for a possible cystine deficiency. Good gains in weight were obtained in all of the eight pairs of rats used with the rations containing approximately 8 per cent of protein, averaging 1.89 gm. daily for the control rats, and 2.09 gm. daily for the cystine-fed rats. In all pairs the cystine-fed rat gained more than the control rat in a feeding period of 56 days, and in all pairs but two gave a greater measurement from nose to anus. The statistical probabilities that this could have been a chance outcome, represented by values of  $P$  of 0.0019 and 0.010, respectively, are much below the critical value of 0.02 to 0.03. It seems clear, therefore, that cystine has exerted a supplementing effect on the proteins of lean beef. This result is in agreement with the experiments of Lewis (6), who showed that the administration of small amounts of cystine to dogs on a diet containing a low percentage of beef heart diminished the loss of nitrogen from the body and favorably influenced the nitrogen balance.

In connection more especially with the problem of the relation between dietary cystine and hair growth and composition, to be discussed in the following paper, it was advisable to repeat the above test on meat protein with a level of meat protein sufficient to support maximum growth. In a preliminary paired feeding test, therefore, a ration containing 18 per cent of meat protein was compared with one containing 20 per cent, and in a second test the 20 per cent meat protein ration was compared with one containing 22 per cent. Only four pairs of rats were used in each test, but the 20 per cent meat protein ration gave larger gains in all cases than the 18 per cent, while the comparison between the 20 and 22 per cent rations was indecisive. Hence, the supplementing effect of cystine on the 20 per cent meat protein ration was tested on eight pairs of rats. The gains on this ration were considerably better than the gains on the 8 per cent meat protein ration, averaging 2.78 gm. daily, while it is evident from the statis-

tics in Table III that cystine has had no appreciable effect on the growth of the rats, a result in sharp contrast with the preceding test on an 8 per cent meat protein level.

*Wheat*—The supplementing effect of cystine on a ration containing 8 per cent of wheat protein was tested on eight pairs of rats. The results possess only a negative significance, since in five of the eight pairs the control rat gained the faster, while in three of the pairs the control rat attained a greater body length. The values of  $P$  for the comparison of gains and of body length, *i.e.* 0.14 and 0.11, are so large that chance alone might well have determined the outcome of the comparison. The average daily gain in body weight (1.37 gm.) was considerably less than in the experiments on meat protein.

While no evidence was obtained for the existence of a supplementing effect of cystine on wheat protein, the results with lysine were clear cut. The lysine was added to rations containing approximately 8 or 10 per cent of wheat protein. The five pairs on the 8 per cent protein ration gained more slowly, averaging 1.03 gm. daily, than the three pairs on the 10 per cent protein ration, *i.e.* 1.57 gm. daily, but in all pairs the lysine-fed rat gained faster than its control. Also, at the end of the experiment the lysine-fed rat had a greater body length than its pair mate in all pairs but one, in which the body lengths were equal. The values of  $P$  for the comparison of the gains in body weight and of the body lengths were so small, *i.e.* 0.0002 and 0.0004, that the significance of the comparisons seems clear.

*Oats*—The possible supplementing effect of lysine on the proteins of whole oats (hulled) was investigated, eight pairs of rats and a basal diet containing approximately 8 per cent of oat protein being used. The rates of gain in a feeding period of 9 weeks averaged 1.30 gm. daily for the control rats and 1.38 gm. daily for the lysine-fed rats. In six of the eight pairs the lysine-fed rat made a greater gain in the same period of time than its control mate on the same amount of food, and in four of the pairs the lysine-fed rat attained the greater body length. The values of  $P$  for the comparisons of gain in weight and of body length are of such size (0.03 and 0.04) that the results are on the border-line of significance. While they strongly suggest a lysine deficiency they cannot be said to establish it definitely. The comparison

of weekly gains shows forty-two favoring the lysine-fed rat, twenty-three favoring the control rat, and seven equal gains. Dividing the latter equally between lysine-fed and control rats, we have forty-five and one-half comparisons favoring the former and twenty-six and one-half comparisons favoring the latter. In 72 comparisons the ideal outcome if chance alone determined it would be thirty-six for both, a deviation of nine and one-half from the observed results. Could chance alone have produced this? The standard deviation of the frequency distribution of the outcome of 72 events, each of which may result with equal probability in either one of two ways, is given by the expression  $\sqrt{0.5 \times 0.5 \times 72}$ , which is equal to 4.2. The deviation of 9.5 from the ideal chance outcome is 2.26 times this standard deviation. Since when  $N$  is as large as 72, the binomial distribution is practically identical with the normal distribution, we may use a table of values of the normal probability integral in the interpretation of this ratio of 2.26. On this basis it appears that chance alone would produce this deviation only once in about forty-two trials, so that this method of analysis indicates rather clearly that the lysine was responsible for the more rapid weekly gains of the lysine-fed rats over their controls.

It is reasonable to expect marked differences in the favorable effects on the growth-promoting values of rations induced by an addition of an amino acid in which the protein of the ration is deficient. In some cases a marked amino acid deficiency may exist, the correction of which will require a comparatively large addition of the amino acid before a second amino acid deficiency becomes manifest. In this case, as was noted for wheat, the supplemented ration will induce much greater gains than the unsupplemented. However, in other cases, a second amino acid deficiency may develop on the addition of a relatively minute amount of the amino acid in which the dietary protein is primarily deficient. In this case, the supplemented ration will be only slightly superior to the unsupplemented in growth-promoting value. This appears to describe the relationship between lysine and the protein mixture in the whole oat kernel.

*Soy Beans*—The proteins of soy beans were tested with reference to a possible deficiency in cystine, a basal ration containing 10 per cent of soy bean protein being used. The results in a feed-

ing period of only 5 weeks were clearly in favor of the supplemented ration. Both rations were consumed in such small amounts that little if any growth occurred, the control rats gaining an average of 2 gm. in 35 days, and the cystine-fed rats an average of 9.2 gm. In spite of this poor growth, the rat fed cystine in each pair gained more than its control mate and attained a greater body length. The values of  $P$  for the body weight gain and the body length comparisons were, respectively, 0.0002 and 0.0003, expressing the extremely small probability that chance could have produced this outcome. The comparison of weekly gains is just as convincing, so that the evidence is reasonably clear that soy bean proteins are deficient in cystine as far as their value in the promotion of growth in the rat is concerned.

*Corn*—In searching for the amino acid deficiencies of corn, a ration containing approximately 8 per cent of corn protein was supplemented first with lysine and then with tryptophane. Lysine evidently exerted a distinct though small supplementing effect. In six of the eight pairs the lysine-fed rat gained faster than its control mate, while in only one pair was the reverse true. In all pairs, the lysine-fed rat at the end of the experiment had a greater body length than its control mate. The differences in total gains between pair mates averaged 3.4 gm. in favor of the lysine-fed rat, and the differences in body length averaged 4.0 mm., favoring the lysine-fed rat. The probabilities that average differences as great as these could have resulted from a chance combination of the innumerable biological factors determining the efficiency of food utilization are, respectively, 0.024 and 0.001. These values are sufficiently small that a good deal of confidence may be felt in the conclusion that chance did not operate alone, and that lysine did in fact exert a supplementing effect. The comparison of weekly gains does not lend any appreciable support to this conclusion, but in this experiment the significance of the weekly gains is considerably impaired by the frequent refusal of feed on the last day of the week. As the figures in the last section of Table III show, the refusal of food was much more frequent among the lysine-fed rats than among their controls.

Tryptophane exerted no supplementing effect on a ration containing 8 per cent of corn protein. In fact in all but one of the eight pairs in the experiment the tryptophane-fed rat gained at a

slower rate than its control mate. The average difference in total gain between pair mates, favoring the control ration, was 2.75 gm. and the corresponding value of  $P$  was 0.0085. This may be considered a significant difference indicating a slight depressing effect of tryptophane upon increase in weight. That this does not mean a depressing effect upon growth is indicated by the purely negative outcome of the experiment with reference to body length. In only four of the eight pairs was the body length of the control rats greater than that of the tryptophane rats, while the average algebraic difference between pair mates was exactly zero.

When tryptophane was added to a ration containing 8 per cent of corn previously supplemented with 0.25 per cent of lysine, a distinct effect was secured, with respect to both total gain and body length. In this case, the tryptophane-fed rat gained more rapidly than its control mate in all pairs, the average difference in total gain being 5.2 gm., with a value of  $P = 0.0051$ . In six of the eight pairs, the tryptophane-fed rat attained the greater body length, the average difference between pair mates amounting to 2.7 mm. with a value of  $P = 0.029$ . The comparison of weekly gains, showing thirty-one favoring the supplemented ration and only seventeen the unsupplemented, affords additional confirmation of the conclusion that the proteins of corn are improved in biological value by the addition of tryptophane after they have been supplemented with lysine. In other words, it may be concluded that the first amino acid deficiency of corn proteins is in lysine, while the second deficiency is in tryptophane.

#### SUMMARY AND CONCLUSIONS

The amino acid deficiencies of the proteins of lean beef, wheat, oats, corn, and soy beans have been investigated, the paired feeding method being used. Eight pairs of rats were used in each test. The results appear to justify the following conclusions.

The proteins of lean beef and of soy beans are biologically deficient in cystine and the deficiency in each case is such that when it is corrected a considerable improvement in the growth-promoting value of the proteins results. The proteins of wheat are deficient in lysine, and, again, the correction of this deficiency results in a large increase in growth-promoting value. The proteins of oats and of corn are also deficient in lysine, but in these

cases a correction of the lysine deficiency results in a distinct but inconsiderable increase in growth-promoting effect. Apparently in these cases a second amino acid deficiency develops after the addition of a minimum proportion of lysine. In the case of corn proteins, the second amino acid deficiency relates to tryptophane. This amino acid added to a ration containing 8 per cent of corn protein exerts no appreciable effect on growth-promoting value, but if added to such a ration previously supplemented with a small amount of lysine, it brings about a distinct increase in growth-promoting capacity.

In the case of tryptophane added to unsupplemented corn proteins, a curious and unexplained depression in rate of gain in weight resulted, slight in magnitude but statistically significant. Since no effect on body length was observed, it can hardly be interpreted as a depression in growth.

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## THE RELATION BETWEEN DIETARY CYSTINE AND THE GROWTH AND CYSTINE CONTENT OF HAIR IN THE RAT

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Relations between hair growth and the endocrine glands have been suggested or established more or less certainly, particularly between hair growth and the sexual glands, the thyroid gland, and more recently the anterior lobe of the pituitary gland (1). Of particular importance to the subject of this paper, however, are those investigations concerned with the effects of the nutrients in the diet upon hair growth and hair composition. Lightbody and Lewis (2) have reviewed a number of investigations which have been interpreted as revealing a relation between the character of the dietary protein and the amount of hair grown. Especially significant is the work of Zuntz and coworkers (3) and of Blaschko (4) on the beneficial effects of keratin preparations on the regeneration of hair in humans and the growth of wool in sheep, although the evidence on this point is not unanimous (5). Lightbody and Lewis themselves have presented evidence indicating that the protein content of the diet affects the growth of hair at levels of protein intake that stunt body growth also. In their second paper they show that the composition of hair with respect to cystine and sulfur may also be affected by the protein level; presumably this effect is due to the variable cystine intake. Beadles, Braman, and Mitchell (6) have established a direct relation between cystine intakes inadequate for maximum growth and the growth of hair, although Barritt, King, and Pickard (7) obtained no indications that cystine added to a normal diet either stimulated the growth

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of rabbit fur or affected its sulfur content. In the last case there was no suspicion that the basal diet was deficient in cystine. Bucciardi (8) has not observed any disturbance of the ratio of labile sulfur to total sulfur in hair due to the feeding of diets poor in cystine. This ratio was affected by age, species, and castration.

#### *Experimental Results*

Some of the paired feeding experiments on the amino acid deficiencies of the proteins of different foods, reported in the preceding paper, were used in a study of dietary factors affecting the growth and cystine content of hair. These experiments were particularly favorable to this study, since the dietary treatment of each pair of rats was the same except for the inclusion in the diet of one rat of a small amount of one amino acid. At the end of the feeding period the rats to be used in this experiment were killed and the hair completely removed from the body by immersing in water at a temperature of 70–72°. After a few minutes immersion the hair was loosened and could be readily pulled out. The hair removed in this way was washed thoroughly with hot distilled water, successive washings being passed through a 100 mesh sieve. It was then transferred to a weighed extraction thimble and dried in an oven. When dry, the thimble and contents were transferred to a Soxhlet extraction tube and extracted continuously for 24 hours, first with absolute alcohol and then with ether. It was then dried in an oven and weighed.

In this way the absolute dry weight of hair was obtained for each rat. The surface area of each rat was then determined from the final body weight and the body length from nose to anus by the formula of Lee (9). The weight of dry hair per sq. cm. of body surface was considered to be the most significant measurement of hair growth. This measurement, of course, takes no account of the hair that may have been shed during the feeding period.

The cystine content was determined in each collection of hair by the colorimetric method of Folin and Marenzi (10). Although this method is not as specific for cystine as the methods of Sullivan (11) or Okuda (12), and frequently gives much higher values than the latter method according to Sullivan and Hess (13), the differences are not considerable in the case of the keratins of hair or

wool. Rimington (14, 15) has shown that practically the entire sulfur content of purified wool and animal hair can be accounted for as cystine, camel hair being a notable exception (16). Although Mueller (17) was able to isolate methionine from wool, it was present only in amounts comparable to those found in sulfur-poor casein.

In a sample of wool containing 3.52 per cent of sulfur, Sullivan and Hess found that the cystine sulfur by the Sullivan method accounted for 97.2 per cent of the total sulfur, by the Okuda method 96.9 per cent, and by the Folin-Marenzi method 106.3 per cent. However, better results with the Folin-Marenzi and even the Folin-Looney methods have been obtained. In a study of the cystine content of seven kinds of wool, Rimington (14) obtained an average of 99.2 per cent of the total sulfur in the cystine as determined by the Sullivan method, and an average of 101.4 per cent of the total sulfur in the cystine as determined by the Folin and Looney method. In a later study of sixteen samples of rabbit fur, Barritt and Rimington (18) obtained an average of 99.7 per cent of the total sulfur in the cystine as determined by the Folin-Marenzi method, the largest individual variation from 100 being one value of 102.7 per cent.

Returning to the experiments to be reported here, the rations, the method of feeding the experimental animals, and other details of the experimental procedure will be found in the preceding article.

The growth of rats on a ration containing 8 per cent of beef protein was markedly stimulated by an addition of cystine. The growth of hair on the unsupplemented and the supplemented rations, in eight pairs of rats, is shown in Table I. In all pairs, the cystine-fed rats exceeded their pair mates in total weight of hair, in weight of hair per sq. cm. of body surface, and in the cystine content of the hair.

The mean difference between pair mates in weight of hair per sq. cm. of body surface was 1.77 mg. in favor of the cystine-fed rat. The standard deviation of differences was 1.04 mg., and the ratio of mean to standard deviation was 1.7. According to "Student's" probability table (19), the probability  $P$  that a mean difference between eight paired observations as large as 1.7 times the standard deviation of differences would result from a fortuitous

## Cystine Influence on Hair

TABLE I

*Effect of a Cystine Supplement to Rations Containing Meat Protein upon Growth and Cystine Content of Hair*

Pair No.	Ration	Body weight	Body length	Body surface	Weight of hair		
					Total	Per sq. cm. body surface	Cystine in hair
8 per cent meat protein							
1	No cystine	137	18.9	246	1.956	7.95	10.3
	Cystine	155	19.3	260	2.705	10.40	13.3
2	No cystine	149	19.2	256	1.915	7.48	10.3
	Cystine	157	19.7	266	2.755	10.36	13.8
3	No cystine	172	20.4	283	2.491	8.80	10.5
	Cystine	184	20.2	285	3.439	12.07	12.9
4	No cystine	165	19.7	270	2.692	9.97	10.1
	Cystine	192	21.0	300	3.703	12.34	13.1
5	No cystine	136	18.5	240	2.126	8.86	10.1
	Cystine	141	19.3	253	2.597	10.26	12.9
6	No cystine	135	18.4	238	2.239	9.41	9.9
	Cystine	140	18.8	246	2.522	10.25	10.3
7	No cystine	142	18.7	246	2.545	10.35	9.3
	Cystine	158	19.5	264	2.864	10.85	13.6
8	No cystine	128	18.0	230	2.056	8.94	10.3
	Cystine	130	18.0	231	2.159	9.35	13.2
20 per cent meat protein							
1	No cystine	157	19.2	259	3.294	12.72	13.2
	Cystine	155	19.1	257	3.088	12.02	13.3
2	No cystine	170	19.9	275			
	Cystine	174	19.9	277			
3	No cystine	165	19.9	273	3.666	13.43	13.4
	Cystine	161	19.9	271	3.520	12.99	13.5
4	No cystine	134	18.3	237	2.866	12.09	13.1
	Cystine	146	19.0	252	3.213	12.75	13.2
5	No cystine	152	19.1	256	3.138	12.26	13.4
	Cystine	146	19.0	252	2.896	11.49	13.4
6	No cystine	144	19.1	252	3.492	13.86	13.4
	Cystine	142	18.6	244	3.192	13.08	13.7
7	No cystine	117	17.8	222	2.374	10.70	13.4
	Cystine	119	17.5	219	2.178	9.95	13.3
8	No cystine	119	17.7	221	2.348	10.62	13.1
	Cystine	121	18.0	226	2.159	9.55	13.2

combination of uncontrolled factors is 0.0014, expressed on a scale of 1 for absolute certainty. Such a small probability, equivalent to one event out of a total of about 700 trials, is so small as to indicate clearly the operation of the one controlled factor; *i.e.*, the presence or absence of added cystine in the diets. It may be concluded, therefore, that cystine added to this diet, the protein of which was deficient in cystine with respect to its growth-promoting potency, stimulated the growth of hair.

The mean difference between pair mates in the percentage of cystine in the hair was 2.8 in favor of the cystine-fed rats. The standard deviation of differences was 1.01, and the ratio of mean to standard deviation, 2.76. In this case  $P = 0.00002$ . It is clear that the cystine supplement has increased the cystine content of the hair in this experiment as well as the amount of hair produced per unit of skin area.

In the preceding article it was shown that raising the content of meat protein in the diet from 8 to 20 per cent eliminated all signs of cystine inadequacy, since an addition of cystine no longer improved the growth-promoting value. The effects of the cystine supplement on the hair produced during subsistence on a 20 per cent meat protein ration is shown in Table I. For one pair of rats, the hair collection was lost. In the other seven pairs there is clear indication that the growth of hair was depressed slightly by the supplement of cystine, six of the pairs contributing to this indication. The mean difference in the weight of hair per sq. cm. of skin surface was 0.55 mg. in favor of the control rats, the standard deviation of differences being 0.51, and the ratio of the two 1.07.  $P$  is equal to 0.013. With respect to the cystine content of the hair, a small average difference between pair mates of 0.086 favored the cystine-fed rats. In this case the standard deviation is 0.112 per cent and the ratio of mean to standard deviation 0.77,  $P$  being equal to 0.041. This probability presents a border-line case, and therefore merely suggests, albeit somewhat strongly, a cystine effect.

If cystine is added to a ration containing a protein mixture not deficient in cystine for the promotion of growth, such as a ration containing 8 per cent of wheat protein, no effect on hair growth or composition is evident. This is evident from Table II. The mean difference between pair mates in the weight of hair per

sq. cm. of body surface is only 0.094 mg. in favor of the cystine-fed rats, the standard deviation being 0.595 mg. and the ratio of the former to the latter only 0.16. The value of  $P$  becomes 0.34, indicating that the outcome might well have been a fortuitous one. The average difference between pair mates with respect to the cystine content of the hair was exactly zero.

Hence, it appears that cystine stimulates hair growth and clearly enhances the cystine content of hair only when the basal

TABLE II  
*Effect of a Cystine Supplement to Ration Containing 8 Per Cent of Wheat Protein upon Growth and Cystine Content of Hair*

Pair No.	Ration	Body weight	Body length	Body surface	Weight of hair		
					Total	Per sq. cm. body surface	Cystine in hair
1	No cystine	128	18.4	235	1.543	6.56	13.3
	Cystine	122	18.2	229	1.593	6.96	13.0
2	No cystine	133	18.2	235	1.562	6.65	13.5
	Cystine	126	18.0	229	1.498	6.54	13.2
3	No cystine	132	18.2	234	1.784	7.63	13.6
	Cystine	124	18.3	232	1.822	7.85	12.8
4	No cystine	135	18.7	242	1.575	6.51	12.7
	Cystine	138	18.7	244	1.833	7.51	13.0
5	No cystine	130	18.5	237	1.747	7.37	12.4
	Cystine	129	18.8	239	1.664	6.96	13.1
6	No cystine	140	18.7	245	1.942	7.93	13.3
	Cystine	151	18.9	253	2.039	8.06	13.6
7	No cystine	131	18.3	235	1.774	7.55	13.4
	Cystine	127	18.5	236	1.920	8.14	13.3
8	No cystine	142	18.6	244	2.025	8.30	13.1
	Cystine	148	19.1	254	1.837	7.23	13.3

ration to which it is added is deficient in cystine for animal growth. What would be the effect on hair growth and composition produced by another amino acid when added to a basal ration deficient in that amino acid? An opportunity to answer this question is found in the experiment described in the preceding paper on the effect of a supplement of lysine added to rations containing 8 or 10 per cent of wheat protein. The effect noted was a marked stimulation of growth. The effect on hair growth is clear from the values given in Table III.

The growth of hair, both absolutely and per sq. cm. of surface area, was markedly greater in the lysine-fed rats than in their controls. The average difference between pair mates in weight of hair per sq. cm. was 1.83 mg. favoring the lysine-fed rat. The standard deviation of differences was 0.98 mg. and  $P$  is 0.0009. On the other hand, the outcome with respect to the cystine content of the hair might well have been determined by chance. The

TABLE III

*Effect of a Lysine Supplement to Rations Containing 8 or 10 Per Cent of Wheat Protein on Growth and Cystine Content of Hair*

Pair No.	Ration	Body weight gm.	Body length cm.	Body surface sq. cm.	Weight of hair		Cystine in hair per cent
					Total gm.	Per sq. cm. body surface mg.	
1	No lysine	126	18.6	236	1.576	6.68	13.6
	Lysine	153	19.2	258	2.162	8.38	13.7
2	No lysine	133	18.7	241	1.695	7.03	14.1
	Lysine	159	19.4	263	2.215	8.42	14.6
3	No lysine	114	17.4	215	1.676	7.80	13.9
	Lysine	125	18.2	231	1.837	7.95	13.2
4	No lysine	135	18.6	241	1.701	7.06	13.3
	Lysine	168	19.4	267	2.212	8.29	13.4
5	No lysine	126	18.4	234	1.700	7.27	14.0
	Lysine	128	18.4	235	2.531	10.77	12.5
6	No lysine	129	18.7	239	1.567	6.55	13.1
	Lysine	155	19.1	257	2.481	9.65	12.9
7	No lysine	128	18.5	236	2.190	9.28	13.3
	Lysine	147	18.7	248	2.690	10.85	12.8
8	No lysine	128	18.3	234	1.916	8.19	13.6
	Lysine	158	19.3	261	2.653	10.17	12.4

mean difference was 0.42 per cent favoring the control rats, the standard deviation 0.64 per cent, and the value of  $P$  0.066.

In the paper of Beadles, Braman, and Mitchell (6) it was shown that a supplement of cystine added to a ration containing 8 per cent of potato protein stimulated body growth and the growth of hair. The samples of hair in this experiment were saved and have since been analyzed for cystine by the Folin-Marenzi method with the results shown in Table IV. Here, as in the case of the basal ration containing 8 per cent of meat protein (Table I), the cystine

content of the hair has been enhanced by the addition of cystine to the diet. The effect is evident in all of the eight pairs, the average difference being 1.77 per cent in favor of the cystine-fed rats, the standard deviation of differences 0.63, and the value of  $P$  less than 0.0001.

It appears that, while any amino acid may stimulate hair growth if added to a basal diet whose general growth-promoting power is thereby increased, an effect on the cystine content of hair results only when a cystine-deficient diet is corrected by a cystine addition. The cystine-deficient diet produces a hair deficient in cystine; the effect is removed by adding cystine to the diet until the cystine deficiency has been satisfied. Hair with a normal

TABLE IV

*Effect of a Cystine Supplement to Ration Containing 8 Per Cent of Potato Protein ( $N \times 6.25$ ) on Growth and Cystine Content of Hair*

Pair No.	Ration	Cystine in hair <i>per cent</i>	Pair No.	Ration	Cystine in hair <i>per cent</i>
1	No cystine	12.2	5	No cystine	12.2
	Cystine	13.5		Cystine	13.6
2	No cystine	12.4	6	No cystine	12.1
	Cystine	14.7		Cystine	15.0
3	No cystine	11.7	7	No cystine	13.1
	Cystine	13.1		Cystine	13.9
4	No cystine	12.1	8	No cystine	11.4
	Cystine	14.0		Cystine	13.6

cystine content is thus produced. Additions of cystine to diets possessing no such deficiency, however, have no certain effect upon hair composition. The production of a hair coat containing a supernormal cystine content does not seem to result from the feeding of excessive amounts of cystine.

#### *Microscopical Studies*

It seems quite inconceivable that the composition of proteins formed by the body during growth should be modified by the nature of the diet fed. The interpretation of these experimental results showing clearly that a cystine-deficient diet will produce a hair growth deficient in cystine must depend rather upon a dis-

turbance of a normal ratio of proteins in the hair fiber, such that there will be a greater proportion of proteins poor in cystine. Microscopically there are two main divisions of the hair fiber; *i.e.*, the cortex and the medulla. The cortex constitutes the main portion of the hair shaft and surrounds the centrally disposed medulla. It is composed essentially of compact keratinized cells, comparable to those of the stratum corneum of the skin, and is translucent. The medulla has a looser structure than the cortex, its cells being fewer, larger, and more loosely held together. Generally among the medullary cells there are spaces filled with gas, probably modified air. The medullary cells, strung along the axis of the hair, may form either a continuous or an interrupted core. In the finer types of wool fiber (merino) there are no medullary cells, the fiber consisting entirely of cortex with a barbed scale covering.

A clue to the problem under discussion is afforded by the interesting and exhaustive studies of Barritt and King and their associates in England on the sulfur content of wool. These investigators have established the existence of a wide variability in the sulfur content of wool, both from type to type and even along the same individual fiber (20). These variations in sulfur content have been associated with variations in the proportion of medullated fibers or in the proportion of medulla in a single fiber. Thus, the coarser kempy type of wool is lower in sulfur than the fine merino type, and correspondingly it possesses a medullated core, while the latter is comprised only of scale and cortex. In the same fleece of blackface wool, the fine non-kempy wool contained 3.82 per cent of sulfur on the dry basis, the coarse kempy fibers 3.33 per cent, and the kelps 3.24 per cent. For the same reason the sulfur content of Welsh mountain lamb's birth coat is very considerably less than that of the wool of the same breed (16), and the puppy coat of rats is less rich in cystine and sulfur than the hair of the adult (2). This type of evidence, leading to the belief that the cortex only is keratinized and hence high in sulfur, is circumstantial in character, but it receives considerable support from some recently published computations of Barritt and King (21). From measurements of the proportion of medulla in certain wool fibers and of the sulfur content of such fibers and of non-medullated wool fibers, it is concluded that "the sulphur content of

medulla substance, if not actually *nil*, must be extremely low in comparison with that of scale and cortex."

In the words of Barratt, King, and Pickard (7), "it appears that the biochemical changes during keratinisation are accompanied by incorporation of sulphur in the case of the scale and cortex cells, but not in that of the medulla cells. This is in agreement with the histological view that the medulla channel is an upward protrusion of the basal epidermal layer. The medulla being sometimes continuous and sometimes intermittent indicates a continuous or intermittent requisitioning of sulphur-free material to make up for a deficiency in production of normal sulphur-containing cortex cells."

In order to ascertain whether the determined differences in the cystine content of hair produced by the feeding of a cystine-deficient diet were accompanied by a difference in structure such as to explain the experimental findings, a microscopical study was made of the samples of hair collected from the sixteen rats on the 8 per cent meat protein ration. Eight of these rats received no supplement, while eight received a supplement of cystine. Two or more slides were prepared from each sample of hair and examined under the microscope with magnifications of 650 to 900.

Among the samples of hair from rats fed upon the cystine supplement the hairs examined, though differing in diameter, possessed a compact medulla, within which the cells were arranged in a regular fashion. Two magnifications of hairs typical of these rats are shown in Figs. 1 and 2.

Among the samples of hair from rats raised on a cystine-deficient diet these normal hairs were also present, evidently making up the greater part of the sample, but in all of these samples occurred hairs of a different type, pictured in Figs. 3 and 4. In these hairs the medullary cells are less regular, less densely packed together and seemingly broader and occupying a greater proportion of the hair shaft. The proportion of cortex is decreased, not only by the broadening of the medullary cells but by the appearance of spaces of considerable size (air cells ?) between the medullary cells. Infrequently, there was a break in continuity of cells, as shown in the lower part of Fig. 4, in the case of the hair transversely disposed and, unfortunately, not in good focus.

In order to put to a test the question whether these differences in hair structure were truly characteristic of the samples of hair

obtained from the rats on a cystine-deficient diet, a series of twenty-one slides containing both types of samples in about equal numbers were given code letters and given to one of the writers (D. B. S.) for microscopic identification. Twenty of these slides were properly identified as being either from a cystine-fed rat or a



FIG. 1



FIG. 2

FIGS. 1 AND 2 Normal rat hairs magnified about 300 and 450 times.



FIG. 4

FIGS. 3 AND 4 Hairs from rats raised on cystine-deficient diets, magnified about 300 times.

control rat, while one slide containing a small sample of hair from a cystine-fed rat was identified as coming from a control rat. In this small sample, no abnormal hair fibers were found. The conclusion is perhaps justified that the abnormal hair structure shown in Figs. 3 and 4 was in fact characteristic of the hair coats produced by the feeding of the cystine-deficient diet.

It seems clear, therefore, that on a diet deficient in cystine the rat will produce hair containing a medulla differing in optical appearance from that of a normal hair, with proportionately broader cells, less regularly disposed, and to all appearances occupying a greater proportion of the fiber. These microscopical findings suggest strongly the conclusion that the lower cystine content of hair produced by the feeding of a cystine-deficient diet is the result of defective keratinization of the hair fiber, which in consequence contains more of the sulfur-poor medullary substance and less of the sulfur-rich cortex.

#### CONCLUSIONS

A ration deficient in cystine inhibits body growth and the growth of hair measured in dry weight per unit of surface. Furthermore, the smaller growth of hair contains a considerably smaller content of cystine than hair produced on the same ration to which a small amount of cystine has been added. This cystine-deficient hair coat, although consisting in the main of hair fibers possessing a normal histological structure, contains a certain proportion of hair fibers whose medullary cells are broader in shape, and loosely packed together. In such hairs the proportion of cortex, representing the completely keratinized part of the hair according to the studies of Barritt and King, is much less than in the normal hair, while the proportion of air cells and medullary cells, consisting of sulfur-poor substance, is correspondingly greater.

While an addition of cystine to a cystine-deficient diet corrects its effects on body growth, hair growth, and hair composition, an addition of cystine to a diet possessing no such deficiency has no certain effect upon hair composition. The production of a super-keratinized hair does not seem to result from the feeding of excessive amounts of cystine. Their effects upon hair growth may indeed be slightly inhibitory.

A ration deficient in lysine inhibits body growth and also the growth of hair, but the cystine content of the hair produced is not lowered.

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## SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM

### VI. STUDIES OF SOME OF THE AMINO ACIDS OF THE YOLK, WHITE, EMBRYO, AND SHELL MEMBRANES DURING DEVELOPMENT OF THE HEN'S EGG

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In the earlier investigations of this series (1-3) the whole developing egg was used but no information was obtained concerning changes in any of the four main fractions. In the present paper a report is made of a study of the tyrosine, tryptophane, cystine, arginine, histidine, and lysine contents of each of the four fractions, namely the yolk, white, embryo, and shell membranes. Such a complete study has not previously been made. An extensive discussion of earlier investigations may be found in (1) and (3) where it will be seen that only one or two of the amino acids have been studied in one or more of the fractions mentioned above.

In this investigation the method of Folin and Ciocalteu (4) was used for the determination of tyrosine and tryptophane, the method of Folin and Marenzi (5) for the determination of cystine, and the author's method (6) for the determination of arginine, histidine, and lysine. The cystine values were corrected for uric acid by the method previously discussed (3).

#### EXPERIMENTAL

Eggs from thoroughbred white Leghorn hens were used throughout this entire investigation. They were incubated at a temperature of 38.5° in a circulating atmosphere which was 84 per cent saturated with moisture. Eggs were removed from the incubator each morning and after careful examination to determine the age of the embryo each egg was separated into the fractions already

mentioned by the method described by Needham (7). Great precautions were necessary at this point to prevent tearing the

TABLE I  
*Distribution of Amino Acids in Various Fractions of Developing Hen's Egg*

	Fresh egg <i>per cent</i>	Day of incubation						
		6th	8th	10th	12th	15th	17th	19th
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
White								
Tyrosine.....	4.26	4.33	4.51	4.41	4.39	4.33		
Tryptophane.....	1.55	1.58	1.45	1.55	1.63	1.62		
Cystine.....	1.67	1.44	1.48	1.33	1.51	1.43		
Arginine.....	5.35		5.47	5.09	5.18	5.01		
Histidine.....	2.14		2.36	2.10	1.91	1.72		
Lysine.....	6.10		6.63	6.47	5.87	6.32		
Yolk								
Tyrosine.....	5.36	5.18	5.02	5.21	5.11	5.02	4.89	4.75
Tryptophane.....	1.61	1.72	1.64	1.68	1.52	1.64	1.67	1.71
Cystine.....	1.28	1.34	1.21	1.37	1.39	1.33	1.35	1.63
Arginine.....	7.65		7.13	7.41	7.10	7.56	7.32	7.49
Histidine.....	1.31		1.27	1.14	1.08	1.10	0.92	0.83
Lysine.....	5.23		5.39	5.41	5.06	5.57	5.09	5.14
Embryo								
Tyrosine.....	5.23	5.06	5.00	4.52	4.37	4.27	4.30	
Tryptophane.....	1.50	1.52	1.58	1.66	1.86	1.89	2.04	
Cystine.....	1.68	1.78	1.84	1.99	2.19	2.38	2.55	
Arginine.....		5.32	5.16	5.42	5.42	5.65	5.38	
Histidine.....		1.99	1.87	1.79	1.70	1.56	1.34	
Lysine.....		6.10	6.40	5.87	5.83	5.95	6.11	
Shell membrane								
Tyrosine.....	4.65	4.58	4.27	4.42	4.33	4.42	4.54	4.47
Tryptophane.....	2.78	2.65	2.64	2.76	2.81	2.68	2.61	2.75
Cystine.....	6.37	6.44	6.31	6.43	6.38	6.24	6.24	6.35
Arginine.....	7.36		7.44	7.10	6.95	7.38	7.27	7.36
Histidine.....	0.58		0.47	0.61	0.49	0.33	0.47	0.48
Lysine.....	2.09		2.14	2.11	2.40	1.99	1.81	2.03

various membranes and admixture of their contents. If admixture did occur the egg was discarded. The fractions were each

then put into boiling absolute alcohol and stirred while the alcohol was again brought to the boiling point. The coagulated material was filtered with suction and extracted in a Soxhlet apparatus with absolute alcohol for 8 hours. It was then washed with ether and dried for 48 hours over sulfuric acid in a vacuum desiccator. The number of eggs used each day usually varied since in the beginning it was necessary to use large numbers in order to get enough dry embryo for analysis and near the end of the period the white was present in small amounts only, so that large numbers of eggs were again required. A total of about 750 eggs was used in this investigation.

TABLE II  
*Ratios of Basic Amino Acids Calculated from Values in Table I*

Period of incubation	Lysine Histidine	Arginine Histidine	Arginine Lysine
<i>days</i>			
Fresh egg	3.62	12.69	3.52
8	4.55	15.83	3.50
10	3.46	11.64	3.37
12	4.89	14.18	2.90
15	6.03	22.26	3.70
17	3.85	15.83	4.01
19	4.22	15.33	3.62
Average.....	4.37	15.39	3.52

The shells, after being thoroughly washed to remove any adhering solute, extracted with alcohol and ether, dried, and weighed, were treated with approximately 1 per cent cold hydrochloric acid to remove the mineral matter from the membranes. The membranes were then filtered off, washed with absolute alcohol and ether, and finally dried in a vacuum desiccator over sulfuric acid. The dried materials of the various fractions consist almost entirely of protein and the values expressed in Table I are not corrected for moisture. The fractions were analyzed by the methods mentioned above and the values expressed in per cent of the weight of the dry material. Analyses were made daily throughout the entire period but only representative days are included in Table I.

## DISCUSSION AND SUMMARY

Only a very few marked changes are noted in the amino acid content of the various fractions of the developing hen's egg. In general the variations are similar to those already noted (2, 3) for the whole egg. The tyrosine decreases in the embryo but remains constant in the other fractions. The tryptophane and cystine increase in the embryo but remain constant in the other fractions. The arginine and lysine remain quite constant throughout while the histidine decreases in all fractions except the shell membranes. The amino acid content of the shell membranes is apparently not influenced by development of the embryo.

Egg-shell membranes are usually considered to be composed chiefly of a keratin and it is interesting to note the ratios of the basic amino acids found in this investigation (Table II) and compare them with the ratios which Block and Vickery (8) have recently considered to be important ratios for the basic amino acids of keratins; namely, 1:4:12 for the histidine, lysine, and arginine respectively.

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## A METHOD TO MEASURE THE TENSION OF CARBON DIOXIDE IN SMALL AMOUNTS OF BLOOD

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This method was designed to measure directly the CO<sub>2</sub> tension of venous blood from isolated gastrocnemius muscles in small dogs. It has been used by Irving, Foster, and Ferguson (1) in the determination of the CO<sub>2</sub> capacity of living mammalian muscle. The principle is that, employed by Krogh and others (2-4), of equilibrating a small volume of air with a relatively large volume of blood, with subsequent analysis of the air. The methods by which this principle had been applied previously were all, for one reason or another, unsuited to our purpose. Moreover, from the viewpoint of general utility, they seemed to leave room for improvement. These considerations led us to attempt the elaboration of this method, which is simple, and easy to apply, and which may be capable of refinement to obtain greater accuracy than we have achieved.

The apparatus consists of two parts (Fig. 1). The first is a combined microtonometer and micro gas analyzer which will be referred to in future as the pipette. It consists of a bulb of 2 cc. capacity blown in glass capillary tubing whose outside diameter is about 5 mm. On one side of the bulb the tubing is 8 to 10 cm. long with an inside diameter of 1 mm. The end is beveled to slide easily into rubber tubing. On the other side of the bulb the tubing is 30 cm. long with an inside diameter of 0.4 to 0.5 mm. At a distance of about 1.5 cm. from the free end, this tube bends at right angles to the axis of the pipette and ends as a small cup, 5 mm. in diameter and depth. The second part is a reservoir for mercury, the capacity of which can be varied at will. It consists of a piece of thick walled rubber tubing about 10 cm. long, into one end of which the short arm of the pipette will fit snugly. The

other end is plugged and the tube is mounted on a metal block in such a manner that it can be compressed by a pair of set screws, one for fine, and the other for coarse adjustments of the volume of the reservoir. Regulation of temperature is obtained by supporting the apparatus horizontally in a large, open water bath equipped with a thermostat. If we had been able to work at room temperature it would have been more convenient to surround the pipette with a water jacket. We had hoped to determine the  $p\text{CO}_2$  at room temperature and calculate the  $p\text{CO}_2$  at 38°, at constant CO<sub>2</sub> content. A little investigation showed that such a procedure would give sufficiently accurate results only over a range of 4 to 5°.

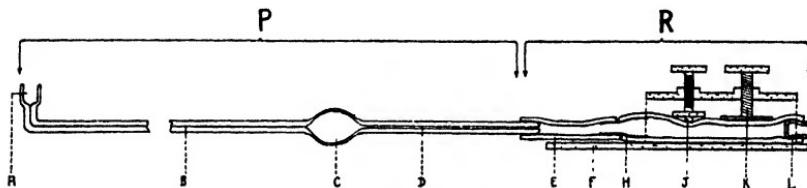


FIG. 1. The pipette is shown connected to the mercury reservoir as it would be when lying in the water bath. *P*, the pipette, comprises microtonometer and micro gas analyzer. *A* is the cup, which is 5 mm. broad and deep; *B*, the fine capillary tube of 0.4 to 0.5 mm. inside diameter, 5 mm. outside diameter, and 30 cm. long; *C*, the bulb of 2 cc. capacity; and *D*, the coarse capillary tube of 1 mm. inside diameter, 5 mm. outside diameter, and 6 to 8 cm. long. *R*, the mercury reservoir is essentially a rubber tube of about 4 cc. capacity, plugged at one end, set on a brass block, and compressible by two set screws. *F* is a brass block; *E*, a rubber tube of 7 mm. outside diameter and 4 mm. inside diameter; *H*, a glass reducing tube to join the narrower rubber tube to a wider one of about 1 cm. outside diameter; *J*, a fine adjustment set screw; *K*, a coarse adjustment set screw; and *L*, a glass plug in the end of the rubber tube.

**Manipulation**—Heparin is used as an anticoagulant. When the ordinary dose of about 14 mg. per kilo is injected into a dog, clotting is prevented for several hours, to all gross appearances, but the formation of small white particles of fibrin which tend to clog the capillary still occurs. About three times the stated dose was found to prevent the formation of these particles. As this is an expensive practice, we have tried recently, with good results, injecting about 20 mg. per kilo into the animal, and in addition drying about 0.003 cc. of 10 per cent heparin solution in the pipette.

A small drop of caprylic alcohol is drawn a few cm. up the short arm of the pipette and blown out again as completely as possible. This is sufficient to prevent the blood from frothing in the bulb. The short arm is now connected to a cannula inserted into a branch of the vein from which blood is to be drawn. Intermittent pressure is applied to the main vein proximal to its point of junction with the branch in which the cannula is inserted, to divert blood into the pipette. When all the air except for a small bubble (the size of which may be gauged by the eye or by a mark on the bulb), has been displaced from the bulb, the pipette is brought nearer to the horizontal, allowing the blood to flow past the bubble which is now trapped in the bulb. When blood has filled the fine capillary the pipette is disconnected and laid in a horizontal position in the water bath. By a little adjustment before the immersion of the pipette, a short column of air can be made to occupy the open end of the coarse capillary, preventing blood and water from mingling. 10 minutes seem to be ample time for equilibrium to become established. During this time the pipette is rolled between the fingers at frequent intervals, to insure mixing.

With the bulb wrapped in gauze soaked in bath water, the pipette is momentarily removed from the bath to be connected with the mercury reservoir. The short arm is dried quickly, and held at such an angle that the blood flows down and fills it completely. The beveled end is slipped into the open end of the reservoir, which has been filled to the brim with mercury. The mercury, which is forced up the coarse capillary, displaces blood into the fine capillary and of necessity disturbs the pressure in the bulb. The pipette and reservoir are returned to the water bath. The blood which has been forced into the cup is mopped out of it. 5 minutes more are allowed for equilibrium to become reestablished before the analysis is begun. When this time has elapsed the bulb is wrapped as before, in wet gauze, and the pipette is brought to a vertical position causing the bubble to lie under the opening of the fine capillary. By turning the coarse adjustment screw the bubble is pushed into the capillary till it occupies a length of 15 to 20 cm. When the pipette is brought again to the horizontal the part of the bubble in the capillary will remain there, confined by two columns of blood. It must be mentioned here that the position in which the pipette reclines in the bath is not

truly horizontal, but slightly tilted to allow the brim of the cup to be well clear of the water while the capillary and bulb are immersed. By turning one or the other of the set screws on the mercury reservoir, the bubble is moved slowly along the capillary till the column of blood separating it from the cup is about 2 cm. long. Now the cup is wiped dry and the bubble moved slowly back into the middle of the capillary. Since the column of blood limiting the bubble distally is only 2 cm. long and since it lies in a practically horizontal, uniform capillary, it has little inertia, and exerts on the bubble practically no pressure above that of the atmosphere. When the total length of the capillary is 30 cm. it is of some advantage to use a bubble not longer than 15 cm.; for then, by moving it slowly up and down the full length of the capillary, all fluid adherent to the walls in excessive amount, because of the somewhat hasty introduction of the bubble, may be removed.

The length ( $l_1$ ) of the bubble is now measured. For this purpose the capillary tubing may be graduated in mm. We have found, however, that the measurement can be made very satisfactorily by laying a steel rule under the tubing. Errors due to parallax can be avoided by looking down into the reflection of your own eye in the water bath. In this way we used home-made pipettes after expensive manufactured ones had been broken. A correction curve was made for each pipette to allow for lack of uniformity in the bore of the capillary.

To absorb the CO<sub>2</sub> from the bubble, a 3 to 4 per cent solution of sodium hydroxide is used. 1 drop of caprylic alcohol is shaken up with about 100 cc. of the solution. This overcomes the tendency of the bubble to break up in the absorber. The bubble is moved along the capillary towards the cup till all but 0.5 cm. of the distal column of blood is in the cup. The latter is wiped out and filled with alkali by means of a medicine dropper. The alkali is drawn into the capillary tube where it mixes with the blood. Since dilute alkali is used, coagulation of the blood with consequent plugging of the capillary need not be feared. The cup, filled to the brim, is inverted. Capillarity keeps the alkali from running out. The pipette must be supported now at an angle of about 15°, that the inverted cup may be clear of the surface of the water. This necessitates a large part of the capillary being out of

water, but that does not matter during the absorption process. The bubble is moved slowly out till it lies almost wholly in the inverted cup. After a pause, it is drawn slowly back into the capillary. Two or three such manipulations suffice to remove all the CO<sub>2</sub>. With the bubble drawn back into the capillary the cup is rotated to an upright position and the tube is reimmersed in the water bath. The alkali is removed from the cup, leaving a column about 2 cm. long in the capillary to confine the bubble. The latter is moved slowly back and forth along the tube till repeated measurements show that it has attained a constant length ( $l_2$ ).  $p\text{CO}_2$  (in mm. of Hg) =  $\frac{l_1 - l_2}{l_1} \times B$  where  $B$  = barometric pressure.

Two analyses can be made with all precautions in 30 minutes or less.

*Sources of Error*—As Krogh (2) has pointed out, it would be impossible for the partial pressure of CO<sub>2</sub> in the bubble to equal that in the blood if the sum of the partial pressures of the gases in the blood did not equal the sum of the partial pressures in the bubble. In the pipette the total pressure of the latter must always be practically atmospheric. The total pressure of gases in the venous blood, however, is nearly always below atmospheric. Its total pressure must be increased by the absorption of nitrogen and oxygen from the bubble. Nitrogen, because of its low solubility coefficient can be absorbed in sufficient quantities to equalize the pressures without diminishing perceptibly the volume of the bubble and presumably, without affecting the CO<sub>2</sub> equilibria in the blood. The absorption of oxygen, however, tends to raise the CO<sub>2</sub> tension. With a bubble whose volume is not greater than 0.06 cc., the available oxygen is about 0.012 cc. If this were all absorbed (which could never quite happen) by 2 cc. of blood, the oxygen content of the blood would rise by 0.6 volumes per cent. From a consideration of the CO<sub>2</sub> dissociation curves of oxygenated and reduced blood it is seen that such an increase in oxygen content could not produce a rise in  $p\text{CO}_2$  greater than 1 mm. of Hg. On the other hand, the loss of CO<sub>2</sub> to the bubble, even when the tension is as high as 150 mm. does not diminish the tension by more than 1 mm. of Hg. The larger the bubble which can be used,

the larger may be the capillary in which it is analyzed. In preliminary experiments we found that the use of a capillary of 0.25 mm. bore was unsatisfactory because it frequently became plugged and often could not be cleaned out. A capillary of 0.4 to 0.5 mm. bore rarely becomes plugged, and in all respects is handier to work with. In a capillary of 0.4 mm. bore a bubble of 0.06 cc. occupies a length of about 47 cm., while in a capillary of 0.5 mm. bore a bubble of that volume occupies a length of 30 cm. A length of 15 to 20 cm. is desirable for each analysis. If it is any shorter, the quantity ( $l_1 - l_2$ ) becomes so small that an error of 0.1 mm. in both  $l_1$  and  $l_2$  will make an error of 2 to 3 per cent in  $(l_1 - l_2)$  when the CO<sub>2</sub> content of the bubble is about 6 per cent.

In addition to the difficulty of temperature regulation, working at 38° presents another disadvantage in that glycolysis becomes a factor of importance. In experiments conducted over a period of 4 or 5 hours it was found that the tension might increase at a rate of 15 to 20 mm. of Hg per hour. However, it does not always increase so rapidly and sometimes does not increase at all.

Two methods of combatting this source of error may be adopted. First, the time of equilibrating and analysis may be cut down to a minimum so that only 20 to 30 minutes elapse between the drawing of the blood and the beginning of the second analysis. This may be done by making the first equilibration last only 5 to 10 minutes. In a large number of cases with dog blood, this procedure seemed to be fairly efficacious, for in less than half the experiments did the second analysis give a higher result than the first. The second method is safer when a speedy determination cannot be made, or when the CO<sub>2</sub> tension is low. It consists of drying enough sodium fluoride solution in the pipette to make a 0.05 per cent solution in the blood. This procedure was effective in all cases where it was tried. Typical experiments are illustrated in Fig. 2, which represents graphically the results of successive determinations made over a period of 4 hours, with and without sodium fluoride.

Loss of CO<sub>2</sub> may occur from the surface of the blood as it is filling the bulb of the pipette. To evaluate this source of error and others which may be considered as systematic, such as the possible effect of allowing the alkali to moisten the capillary, the method was compared with a macrotonometric method. 5 cc. of blood (usually human) containing sufficient heparin (about 2

mg.) and 1 drop of caprylic alcohol were placed in a tonometer of 400 cc. capacity. The latter was flushed with a 6 to 8 per cent mixture of  $\text{CO}_2$  in air. Half an hour was allowed for equilibrium to become established, during which time the tonometer was kept rotating. The blood was then collected in a well at the bottom of the tonometer whence it was drawn by suction through a tap

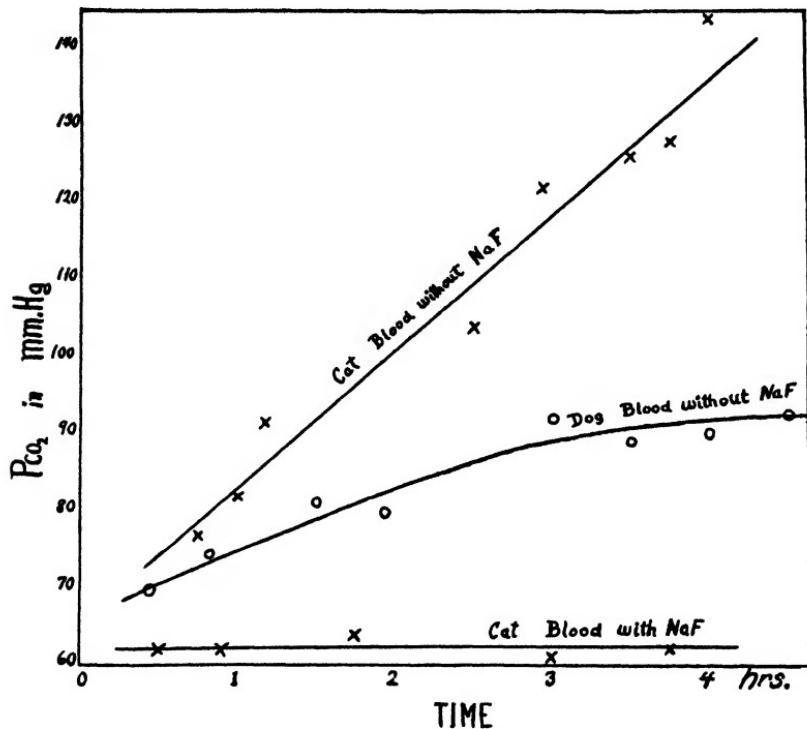


FIG. 2. This diagram illustrates how  $\text{CO}_2$  tension may rise (presumably because of lactic acid formation by glycolysis) in blood kept at constant  $\text{CO}_2$  content at  $38^\circ$ . It shows too the efficacy of adding sodium fluoride (0.05 per cent) to prevent rise in  $\text{CO}_2$  tension.

into the pipette. From a tap at the other end of the tonometer a sample of gas (about 30 cc.) was taken into a Brodie sampling pipette and later analyzed for  $\text{CO}_2$ . The equilibration was done in the macrotonometer at room temperature in a gas analysis room where changes in temperature were relatively slight ( $0.5^\circ$  per hour). The pipette, however, was surrounded by a water jacket

CO<sub>2</sub> Tension of Blood.

containing water at room temperature. The results are given in Table I. The average of the positive and negative discrepancies in fourteen consecutive experiments shows that the micro-

TABLE I

The CO<sub>2</sub> tension of blood which had been equilibrated with a CO<sub>2</sub> mixture in a large tonometer was determined in a microtonometer and compared with the tension of CO<sub>2</sub> in the large tonometer as measured in a Haldane gas analyzer. Fourteen consecutive experiments are reported below.

Date	CO <sub>2</sub> tension in large tonometer (gas analysis in Haldane apparatus)	CO <sub>2</sub> tension in small tonometer		
		1st analysis	2nd analysis	3rd analysis
1890	mm. Hg	mm. Hg	mm. Hg	mm. Hg
Aug. 12	52.8	51.7	50.3	51.7
" 13	52.6	51.7	50.0	51.6
" 14	53.7	55.2	55.4	60.7
" 15	57.2	58.2	58.2	57.3
" 16	58.6	60.3	54.5	
" 18	51.0	48.3	48.2	
" 20	65.0	59.1	60.8	
" 20	49.1	50.0		
" 21	50.4	44.3	43.2	
" 21	53.8	50.7	51.2	
" 22	49.7	43.8	47.7	51.3
" 26	54.8	51.4	52.0	
" 27	58.5	59.3	63.0	
" 29	57.5	54.7	57.7	
Average.....	54.6	52.8	53.2	54.5

Though the figures in each column are not measurements of the same quantity, the averages for each column are calculated because they show at a glance whether the microtonometric determinations tend to be higher or lower than those from the large tonometer. (The difference between the mean of each of the three right-hand columns and that of the first is numerically equal to the mean of the positive and negative deviations of the measurements in each of the small tonometer columns from those in the large tonometer column.)

tonometer gives a result 1.6 mm. lower than the macrotonometer. This can hardly be regarded as a significant difference.

To evaluate the probable error of a single determination on the microtonometer, under the conditions in which it is being used in practice, two methods were employed. In the first way several

pipettes were filled with blood from a glass syringe, in which the blood had been well mixed and protected against glycolysis by addition of sodium fluoride. Two determinations were made on each pipette so that a series of about eight figures, which should have the same value, were obtained and from which a mean, average deviation, and probable error could be calculated. The probable error was taken to be  $0.845 \times$  (the average deviation of a single determination). A series of twenty such determinations gave a probable error of 1.2 mm.

An idea of the probable error may be obtained too from the results of the animal experiments in which a large number of tensions varying from 18 mm. to 120 mm. have been measured with duplicates on each sample. In this series of 72 consecutive determinations, fluoride was not used, glycolysis being left as an added source of error.

(The average discrepancy, in mm. of Hg, of duplicate measurements)  $\div 2$  is taken as the average deviation of a single determination. Probable error = 0.845 (average deviation). The probable error of a single determination calculated in this manner = 1.98 mm. of Hg.

When two pipettes were filled with venous blood from the same muscle, another source of error was included; *viz.*, the possibility of change in the CO<sub>2</sub> tension of the venous blood in the few minutes which necessarily elapsed between filling of the first and second pipette. For this reason these figures were kept separate and constitute another series of thirty-two consecutive experiments. The probable error calculated from this series is 1.7 mm. of Hg, indicating that the added source of error could not have been very important. From the foregoing we may conclude that the probable error of a single determination is less than 2 mm. of Hg in these experiments.

#### SUMMARY

A method to measure directly the tension of carbon dioxide in 2 cc. of blood is described. The principle is that of equilibrating a bubble of air with the blood and analyzing the bubble in a capillary tube. One instrument, the pipette, includes both analyzer and microtonometer. In the analyzer the bubble is confined by blood. CO<sub>2</sub> is absorbed with 3 to 4 per cent NaOH. The prob-

able error of a single determination made as it was in a large number of experiments, is less than 2 mm. of Hg. With certain precautions it may be reduced to about 1.2 mm. of Hg.

In conclusion I wish to express my gratitude to Dr. Laurence Irving for his advice and encouragement, and to thank Mr. H. C. Foster, who made many of the determinations, of which the results are used in this paper.

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## SPINASTEROL AND SOME OF ITS ESTERS

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Clenshaw and Smedley-MacLean (1) showed that less than 5 per cent of the unsaponifiable material of spinach fat was of the nature of a sterol being precipitable by digitonin. In a previous publication (2) from this laboratory, we have described the isolation of the sterol from spinach fat. This sterol, spinasterol, melted at 165–167° and analyzed for  $C_{27}H_{46}O$ .  $[\alpha]_D^{25} = +1.7^\circ$ . This formula ( $C_{27}H_{46}O$ ) was confirmed by the work of Collison and Smedley-MacLean (3). The Liebermann-Burchard test was positive and the Salkowski reaction was similar to that of ergosterol. The Rosenheim reaction (4) for the  $\Delta^{1,2}$  (or  $\Delta^{1,13}$ ) double bond in sterols was negative. No evidence of the inactive (to hydrogenation)  $\Delta^{10,19}$  linkage was obtained by the Tortelli-Jaffe reaction as modified by Heilbron and Spring (5); also spinasterol could be easily reduced, with Adams' (6, 7) platinum oxide catalyst in the presence of hydrogen, to the saturated derivative spinastanol, the chemistry of which will be reported on in a later paper from this laboratory.

In order to provide additional material for a more extensive study, further quantities of spinach were worked up for spinasterol. The purified spinasterol melted at 168–169° and  $[\alpha]_D^{25} = -1.8^\circ$ .

For the purpose of a more exact characterization of spinasterol, a series of the more common esters was prepared.

### EXPERIMENTAL

450 kilos of dried spinach were exhaustively extracted with acetone. The acetone was removed *in vacuo* and the residue dissolved in petroleum ether, and filtered when cold to separate chlorophyll. The chlorophyll thus obtained was saponified, and

from the unsaponifiable fraction was isolated a small amount of material, which after several crystallizations from aqueous pyridine, melted at 283°. This material gave a positive Liebermann-Burchard color test and was a phytosterolin.

The petroleum ether-soluble material was separated by crystallization from alcohol into two fractions: an alcohol-soluble fraction and an alcohol-insoluble fraction.

The alcohol-soluble fraction was saponified by 10 per cent alcoholic potash and the unsaponifiable fraction obtained in the usual manner. The residue from the ether extraction, on crystallization from a mixture of alcohol and ether, yielded 143 gm. of crude unsaponifiable material.

The alcohol-insoluble fraction was saponified and the unsaponifiable material obtained in a similar manner. This unsaponifiable fraction consisted largely of a mixture of sterols and hydrocarbon, difficult to separate by fractional crystallization.

*Fractionation of Crude Unsaponifiable Fraction*—The 143 gm. of the crude unsaponifiable material were crystallized from hot alcohol. The crystalline material filtered off at 60° consisted of crude spinasterol melting at 162–164°. The filtrates from the crude spinasterol on concentration yielded fractions melting at 90–145°.

The fractions melting at 90–145° on treatment with ether were easily separated into ether-insoluble and ether-soluble material. The ether-insoluble material consisted of 5.66 gm. of crystalline material melting at 102–103°. The ether-soluble fraction was a mixture of a more soluble sterol than spinasterol, with hydrocarbon and alcohols. The results of the study of this more soluble material isolated in the filtrate from spinasterol will be reported on in a later paper from this laboratory.

*Spinasterol*—The crude spinasterol was systematically fractionated from ether. The first eight fractions crystallized in flat hexagonal plates that melted at 168–169° with  $[\alpha]_D^{23} = -1.8^\circ$ . The total yield of the pure spinasterol was 45.66 gm.

*Spinasterol Acetate*—Spinasterol was boiled with an excess of acetic anhydride. The resulting crystals of spinasterol acetate melting at 177–181° were crystallized once from acetic anhydride and twice from acetic acid. Hexagonal plates were obtained melting at 183–185° and with  $[\alpha]_D^{23} = -4.7^\circ$ .

$C_{28}H_{48}O_2$ . Calculated. C 81.2, H 11.3  
Found. " 81.4, " 11.2

On saponification, spinasterol of correct melting point was obtained.

*Spinasterol Benzoate*—Benzoyl chloride (2.25 gm.) was slowly added in the cold to 1.5 gm. of spinasterol in 30 cc. of pyridine and then heated on the steam bath for 90 minutes. The pyridine solution was cooled, poured into dilute sulfuric acid (1–5), cooled with ice, and the resulting precipitate extracted with ether. The residue from the washed and dried ether extract after crystallization from ether melted at 198–200°. After further crystallization from ethyl acetate the benzoate was obtained in the form of hexagonal plates melting at 201–202°.  $[\alpha]_D^{23} = +2.25^\circ$ .

$C_{34}H_{50}O_2$ . Calculated. C 83.2, H 10.3  
Found. " 83.5, " 10.1

On saponification, spinasterol melting at 167–169° was obtained.

*Spinasterol Phenylurethane*—This derivative was made by refluxing for 4 hours 1 gm. of spinasterol with 2.5 cc. of phenylisocyanate in 50 cc. of dry benzene (8, 9). The solution was evaporated to dryness and the residue crystallized once from alcohol and seven times from ethyl acetate. The phenylurethane of spinasterol was obtained as flat plates melting at 173–174°.  $[\alpha]_D = -2.25^\circ$ .

$C_{34}H_{51}O_2N$ . Calculated. C 80.7, H 10.2, N 2.8  
Found. " 80.5, " 10.2, " 2.8

On saponification, spinasterol melting at 167–169° was obtained.

*Isospinasterol Chloroacetate*<sup>1</sup>—Spinasterol (1 gm.) was refluxed for 15 minutes with a slight excess of chloroacetyl chloride. The resulting crystals after several crystallizations from ethyl acetate

<sup>1</sup> The reaction of spinasterol and chloroacetyl chloride buffered in pyridine solution gave a halogen-free compound melting at 206–208°, as well as a yield of over 30 per cent of unchanged spinasterol.

Spinasterol fused with chloroacetic anhydride gave the isomeric spinasterol chloroacetate melting at 155–156° with  $[\alpha]_D = -6.0^\circ$  and giving on saponification isospinasterol melting at 148–150° with  $[\alpha]_D^{23} = +5.2^\circ$ .

The study of isospinasterol is being continued in this laboratory and will be reported on in a later paper.

formed flat hexagonal plates melting at 155–156°.  $[\alpha]_D^{23} = -6.0^\circ$ .

$C_{29}H_{47}O_2Cl$ . Calculated. C 75.2, H 10.2, Cl 7.7  
Found. " 75.4, " 10.3, " 8.0

Preparation of the chloroacetate by this method resulted in the isomerization of the spinasterol as the sterol recovered after saponification of the ester melted at 148–150° with  $[\alpha]_D^{23} = +5.2^\circ$ .

*Spinasterol-p-Nitrobenzoate*—This derivative was made in a similar manner to the benzoate. The residue from the ether extract after two crystallizations from ethyl acetate gave felted needles of spinasterol-p-nitrobenzoate melting at 217–218°.  $[\alpha]^{23} = +4.5^\circ$

$C_{24}H_{44}O_4N$ . Calculated. N 2.6. Found. N 2.8

On saponification, spinasterol-p-nitrobenzoate gave spinasterol melting at 167–169°.

*Spinasterol Trichloroacetate*—This ester was made by a similar method used for the preparation of spinasterol benzoate. After one crystallization from acetic acid the melting point was 161–167°. Five more crystallizations from ethyl acetate and one from acetone gave flat plates melting at 167–169°.  $[\alpha]_D^{23} = -3.5^\circ$ .

$C_{29}H_{46}O_2Cl_3$ . Calculated. Cl 20.0. Found. Cl 19.5

On saponification, spinasterol trichloroacetate gave spinasterol melting at 167–169°.

*Spinasterol Propionate*—This derivative was prepared by the reaction of spinasterol with the acid chloride in pyridine solution. Crystallized from ethyl acetate the propionate formed flat plate-like crystals melting at 152–153°. Three more crystallizations failed to raise this melting point.  $[\alpha]_D^{23} = -5.0^\circ$ .

$C_{30}H_{50}O_2$ . Calculated. C 81.4, H 11.4  
Found. " 81.4, " 11.1

On saponification, spinasterol propionate gave spinasterol melting at 167–169°.

*Spinasterol Butyrate*—This derivative was prepared in a similar manner to the benzoate. After crystallization once from acetic acid, twice from ethyl acetate, and once from a mixture of alcohol

and acetone the butyrate formed flat plates melting at 131–132°.  
 $[\alpha]_D^{23} = -4.0^\circ$ .

$C_{31}H_{52}O_2$ . Calculated. C 81.5, H 11.5  
Found. " 81.6, " 11.3

On saponification, spinasterol butyrate gave spinasterol melting at 167–169°.

#### SUMMARY

1. Spinasterol and the following esters were prepared and described: acetate, benzoate, phenylurethane, *p*-nitrobenzoate, trichloroacetate, propionate, and butyrate.
2. Chloroacetyl chloride reacting on spinasterol in the absence of a buffering substance such as pyridine causes isomerization to take place with the formation of isospinasterol chloroacetate.
3. Colorimetric tests indicate the absence of the  $\Delta^{1,2}$  (or the  $\Delta^{1,13}$ ) and the inactive  $\Delta^{10,19}$  linkages in spinasterol.

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## CONCENTRATION OF VITAMINS B<sub>1</sub> AND B<sub>2</sub>

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The present communication contains a report on the results of several years work on the problem of the concentration of vitamin B, an investigation which was, however, discontinued by us in the spring of 1930. A review of the literature on the subject will not be given here, inasmuch as it may be found in many publications of other writers and particularly in the excellent monograph by Sherman and Smith.<sup>1</sup>

*Normal Growth of White Rat at Different Periods of the Year*—In the course of the present investigation it was observed on several occasions that the growth curve produced by a given sample of material could not be duplicated. In particular, indications were obtained suggesting that the identical material seemed to produce better growth early in the fall than later in the year. These observations caused us to undertake the study of growth from month to month, produced by a normal diet. Each new experiment was initiated on the 1st day of each month.

Four animals weighing 50.0 gm. were taken for each experiment and the weights of the animals were observed on the last day of each month. The results are given in Table I.

From Table I it may be seen that the rates of growth for the months of July and August were abnormally low, whereas the rate for September was abnormally high. The climatic conditions of New York during these 2 summer months afford an explanation of this abnormally low growth. It is possible that the abnormally high growth for September is due to a compensatory mechanism, inasmuch as the average increase in weight (52 gm.) per month

<sup>1</sup> Sherman, H. C., and Smith, S. L., *The vitamins*, 2nd edition, New York (1931).

for the 5 exceptional months is the same as the average increase per year.

On the basis of these findings the rate of growth of the experimental animals when placed on the vitamin diet was compared not with the average rate of growth per year but with the normal growth for the month of the experiment.

TABLE I  
*Increase in Weight on Normal Diet at Different Seasons*

Month	Rat 1	Rat 2	Rat 3	Rat 4	Average increase
	gm.	gm.	gm.	gm.	gm.
Sept.	76	89	85	89	85
Oct.	58	59	62	58	59
Nov.	59	60	58	58	59
Dec.	53	53	51	53	53
Jan.	55	53	54	56	57
Feb.	46	45	47	48	47
Mar.	49	49	47	47	48
Apr.	48	46	45	44	46
May	55	59	59	59	58
June	46	54	56	54	53
July	34	30	53	34	33
Aug.	24	25	25	24	25
Average per month for year.....					52
" " " " July, Aug.....					29
" " " " " Sept., Oct.....					72.0
" " " " " " Nov.....					59.0

*Relationship between Growth and Quantity of Dried Yeast Added to Standard Diet*—Brewers' yeast<sup>2</sup> was the material used for the preparation of vitamin extracts.

The activity of the acetone precipitate from dry yeast was used as a standard for comparison with that of the various extracts and concentrates. Only those fractions that were capable not only of maintaining normal growth but also of producing growth above normal were considered to contain all the factors present

<sup>2</sup> The yeast was furnished to us by the Jacob Ruppert Brewery. We take this occasion to express our appreciation to the management for their courtesy.

in the dry yeast, inasmuch as dry yeast possessed this faculty if given in sufficient doses.

This capacity is illustrated in Table II.

Thus, the rate of growth in October as compared with the normal was 1.4:1 in 24 days on 0.300 gm. of dry yeast per day and 1.7:1 on 0.400 gm. In April, on addition of 0.300 gm., the rate of growth as compared with the normal was 1.5:1.

TABLE II

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Yeast*

Month	Time	Normal increase	Yeast added per day		
			0.200 gm.	0.300 gm.	0.400 gm.
	days	gm.			
Oct.	24	46		62	80
"	30	59		75	
Apr.	30	58	64	90	
"	100		135	185	

*Effect of Material Obtained from Alcoholic Extract of Brewers' Yeast*—Three forms of crude vitamin material were used: A, alcoholic extract; B, press-juice; C, precipitate obtained from the latter by means of acetone. Each one of the materials was tested for its capacity to produce normal and accelerated growth.

*A, Alcoholic Extract*—The following figures represent the average increase in weight in 30 days.

Month	Time	Normal increase	Increase in weight in gm.						
			Crude vitamin added per day, gm.						
			0.007	0.010	0.020	0.030	0.040	0.050	0.060
Nov.	days	gm.	0	29	42	65	76	82	96

Thus, it would seem that 0.030 gm. of crude vitamin per day was sufficient to bring about a normal growth and that higher doses accelerated the rate of growth in proportion to the increase in the daily doses of the active material. On 0.060 gm. per day the ratio to normal growth was 1.7:1, which was the same as on 0.400 gm. of the dry yeast.

*B, Press-Juice*—Table III represents the results of the experiments with press-juice.

This material seems more potent than the alcoholic extract, inasmuch as 0.0063 gm. per day sufficed to maintain normal growth. On a daily dose of 0.0175 gm. the ratio between the treated and normal was 1.8:1, and on a daily dose of 0.125 gm. the ratio was 2.42:1.

TABLE III

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Press-Juice*

Month	Time	Normal increase	Yeast juice added per day, cc.						
			0.05	0.075	0.1	0.15	0.25	0.5	1.0
			Dry residue added per day, gm.						
days	gm.		0.0063	0.0088	0.0125	0.0175	0.026	0.0513	0.125
Apr.	18	26	26	32	38	52	45	53	63
"	24	35	33	43	47	63			

TABLE IV

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Acetone Precipitate Obtained from Press-Juice*

Month	Time	Normal increase	Acetone precipitate added per day, gm.						
			0.005	0.0070	0.010	0.015	0.025	0.050	0.100
			days	gm.					
Apr.	18	26					45	40	
"	24	35			48	59		52	56
May	12	22	22	27					

*C, Acetone Precipitate*—Table IV represents the results of the experiments with the acetone precipitate obtained from press-juice.

Comparing the ratios of growth on administration of materials B and C, it is evident that the acetone precipitate retains nearly the total efficiency of the press-juice. Thus, on a daily dose of 0.05 gm. the ratios are 2:1 in both cases.

*Concentration of Crude Vitamin Material by Means of Silica Gel\**

*Preparation of Material*—100 gm. of the crude material were triturated in 4 liters of water. Part remained undissolved and was separated from the aqueous part by filtration. To the filtrate 800 gm. of silica gel were added with stirring, and the mixture was maintained at pH 3. After 10 minutes the silica gel was filtered off and washed with very small portions of water. The operation was repeated twice. The silica from the three extractions was combined and suspended in 6 liters of water and to it a solution of lithium hydroxide was added until pH 9.8 was attained. After stirring for 20 minutes, the mixture was filtered, the filtrate rendered neutral by means of a solution of hydriodic acid, and

TABLE V

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Silica Concentrate (Concentrate 159)*

Month	Time	Normal increase	Concentrate added per day, gm.								
			0.003	0.006	0.007	0.010	0.020	0.030	0.040	0.050	0.060
.	days	gm.									
Apr.	12	16	19	22	26	28	33	37	37	43	56
"	18	26		31	32	39	44	53	53	59	73
"	24	35		38	45	51	55	71	71	73	89
"	30	46		47	55	57	63	85	84	88	106

then concentrated to 100 cc. It was allowed to stand in the refrigerator until precipitation of the small amount of silica gel still remaining in suspension was completed. A small portion of alcohol was then added and the mixture filtered. The filtrate was further concentrated and acetone was added to the concentrate until precipitation was completed. The yield of the dried product was about 18 to 20 gm. The dried precipitate was tested for its efficiency, and the results are given in Table V.

Thus, as compared with the crude vitamin preparation, the minimal dose required to bring about normal growth was in the ratio of 3:25. The material was not improved, however, in the same degree with respect to producing a growth above normal. On daily doses of 0.050 gm. the ratio was as 1.4:1.8, and on 0.060

\* We wish to express our appreciation to the Silica Gel Corporation for their generosity in supplying the silica gel for this work.

gm. as 1.6:2.3. This fact may be explained by assuming that the silica concentrate contained in different proportions the several factors necessary for growth.

In addition, more effective material was prepared through the agency of silica gel when, instead of the alcoholic extract, press-juice was used as starting material, thus showing that the extrac-

TABLE VI

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of the Silica Concentrate Obtained from Press-Juice*

Month	Time	Normal increase	Silica concentrate added per day, gm.	
			0.002	0.005
May	days	gm.		
	12	22	23	37
	18	30	34	52
"	24	43	45	

TABLE VII

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of the Purified Silica Concentrate (Concentrate 540 Obtained from Press-Juice)*

Month	Time	Normal increase	Concentrate added per day, gm.	
			0.002	0.005
May	days	gm.		
	12	22	22	37
	18	33	34	51
"	24	44	45	

tion with dilute alcohol is to some extent deleterious to some of the factors contained in the growth-promoting material.

From Table VI it may be seen that daily doses of 0.005 gm. of this concentrate produced a growth higher than normal in the ratio of 1.7:1, while the same dosage of the concentrate prepared from the alcoholic extract was only capable of maintaining normal growth.

*Further Concentration of Material Described Above*—The material prepared by silica absorption was further concentrated in the

following manner. 3.0 gm. of the dry powder were dissolved in 10.0 cc. of water and to this solution were added 100 cc. of ethyl alcohol containing 1 cc. of 70 per cent hydriodic acid. The precipitate thus formed was centrifuged and then washed with alcohol until the washings were free of iodide ions. The precipitate contained the active material, whereas the material obtained from the filtrate was inactive. The results are shown in Table VII.

Thus, when administered alone this material did not possess greater potency than the original material.

Many other ways of concentrating the material absorbed by silica gel were tried, but most of the preparations obtained by the various methods were found to be effective for only relatively short intervals (of 1 or 2 weeks) after which growth proceeded at a very low rate. These failures naturally suggested the possibility of the existence of more than one factor contained in the original growth-promoting material. The existence in the yeast of two factors, one antineuritic and the other growth-promoting, was suggested some time ago by Emmett and Luros,<sup>4</sup> Funk and Dubin,<sup>5</sup> and Levene and Muhlfeld.<sup>6</sup> It was, however, Goldberger and his associates<sup>7</sup> and Chick and Roscoe<sup>8</sup> who made the important discovery that one factor (the antineuritic according to Goldberger) was heat-unstable ( $B_1$ ), whereas the second (antipellagra) was heat-stable ( $B_2$ ). These discoveries were made at a time when our own experiments had convinced us that our purified silica concentrates were lacking in some of the factors required for maintenance of normal growth. The findings of Goldberger furnished a method for detecting which of the two factors predominated in the purified silica concentrate. Indeed, it was established that this material consisted in the main of the heat-labile antineuritic fraction. The solution remaining after the silica extraction should furnish the source for the heat-stable factor. This expectation was actually realized.

<sup>4</sup> Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, **43**, 265 (1920).

<sup>5</sup> Funk, C., and Dubin, H. E., *Proc. Soc. Exp. Biol. and Med.*, **19**, 15 (1921-22).

<sup>6</sup> Levene, P. A., and Muhlfeld, M., *J. Biol. Chem.*, **57**, 341 (1923).

<sup>7</sup> Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U.S.P.H.S.*, **41**, 297 (1926).

<sup>8</sup> Chick, H., and Roscoe, M. H., *Biochem. J.*, **21**, 698 (1927). See also Narayanan, B. T., and Drummond, J. C., *Biochem. J.*, **24**, 19 (1930).

*Vitamin B<sub>1</sub> Potency of Material Prepared from Silica Concentrate*—In order to determine the content of factor B<sub>1</sub> in the silicon

TABLE VIII

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Fractions B<sub>1</sub> and B<sub>2</sub>,*

Fraction B<sub>2</sub> was from Concentrate 447.

Month	Time	Normal increase	B <sub>2</sub> , 0.030 gm. per day (constant); B <sub>1</sub> (Concentrate 519) added per day (variable)	
			0.0002 gm.	0.0005 gm.
	days	gm.		
Feb.	12	18	24	33
"	18	27	32	44
"	24	36	38	53
"	30	47	50	62
			B <sub>1</sub> (Concentrate 150), 0.0002 gm. per day (constant); B <sub>2</sub> added per day (variable)	
			0.040 gm.	0.050 gm.
Feb.	12	18	21	23
"	18	27	30	32
"	24	36	38	42
			B <sub>2</sub> , 0.060 gm. per day (constant); B <sub>1</sub> (Concentrate 150) added per day (variable)	
			0.003 gm.	0.005 gm.
Feb.	12	18	23	32
"	18	27	32	45
"	24	36	40	54
			B <sub>1</sub> (Concentrate 519), 0.0001 gm. per day (constant); B <sub>2</sub> added per day (variable)	
			0.020 gm.	0.040 gm.
Apr.	12	16	14	19
"	18	26	18	27
"	24	35	21	34
			0.050 gm.	0.060 gm.

concentrate, daily doses of this concentrate were added to crude material which had been heated to 140° in an autoclave, a procedure which destroyed the heat-labile factor B<sub>1</sub>, leaving the factor B<sub>2</sub> intact. In preliminary experiments it was found that 0.030 gm. of this material was a sufficient daily dose of factor B<sub>2</sub>.

Table VIII contains the results of several such experiments, from which it will be seen that when a sufficiently large dose of the heat-stable material was added to the diet, less than 0.0001 gm. of the B<sub>1</sub> concentrate sufficed to maintain normal growth. It may also be seen that with higher daily doses of the B<sub>1</sub> concentrate, a growth rate higher than normal can be attained. However, the minimum dose has to be multiplied many times before a growth rate above normal can be obtained, whereas increasing the dry yeast by about 50 per cent of the minimum requirement results in a 50 per cent increase in the rate of growth. Is this an indication that the sum of the purified silica concentrate and the heated material does not contain all the factors present in the crude material, or does it indicate that the factors B<sub>1</sub> and B<sub>2</sub> are impaired in some of their activities? These questions cannot as yet be answered.

It must be emphasized that the values given for normal growth are the averages of four experiments each. The values given for the concentrated materials (Concentrates 159 and 519) are representative of samples of a number of experiments. Occasionally it was found that the materials obtained in exactly the same manner were less potent than those discussed; as a rule, however, the materials were of the same degree of potency. All samples were, of course, very complex mixtures and all contained in the neighborhood of 25 per cent of mineral matter, including the phosphoric acid bound in ester form (mainly nucleic acid derivatives). The composition of Concentrate 159 was C 34.71, H 5.36, N 6.80, and P 3.00. Calculated as ash-free material, the composition was C 39.85, H 6.15, N 7.80, and P 3.44. Several other samples had approximately the same composition.

At this phase of the work, further purification and concentration of the heat-labile fraction was interrupted and attention was directed to the concentration of the heat-stable fraction.

#### *Concentration of Fraction B<sub>2</sub>*

This material was obtained from the solution remaining after extracting the solution of the crude material with silica gel. Repeating the extraction with silica gel six times leaves a solution which, after neutralization with lithium hydroxide, concentration, and precipitation with acetone, yields a precipitate (Concentrate

546) which is totally inactive when given by itself. Combined with the heat-labile fraction, in doses incapable by themselves of maintaining growth, it leads to normal growth. (See Table IX.)

3.0 gm. of Concentrate 546 were then dissolved in 10 cc. of water and allowed to flow slowly into alcohol containing 0.5 cc. of a 70 per cent hydriodic acid solution. A precipitate formed which was washed with alcohol until the washings no longer contained hydriodic acid (Concentrate 547). (See Table X.)

TABLE IX

*Average Increase in Weight (in Gm.) of Rats on Administration of Purified Fractions B<sub>1</sub> and B<sub>2</sub>*

Fraction B<sub>1</sub>, 0.0002 gm. of Concentrate 519 per day (constant).

Fraction B<sub>2</sub>, Concentrate 546 in increasing doses per day (variable).

Month	Time	Normal increase	B <sub>2</sub> added per day, gm.	
			0.005	0.007
May	days	gm.	.	
	12	22	22	24
"	18	34	32	35

TABLE X

*Average Increase in Weight (in Gm.) of Rats on Administration of Purified Fraction B<sub>1</sub> and Further Purified Fraction B<sub>2</sub>*

Fraction B<sub>1</sub>, 0.0002 gm. per day (constant).

Fraction B<sub>2</sub>, Concentrate 547 in increasing doses in gm. per day (variable).

Month	Time	Normal increase	B <sub>2</sub> added per day, gm.				
			0.0005	0.001	0.002	0.003	0.005
June	days	gm.					
June	12	20	19.0	24	25	30	37

Each value is the average of several experiments. At this stage the work was discontinued. It is now being published in the belief that the materials of the type of Concentrates 519 and 547 may serve as starting material for further concentration of the growth-promoting principle and also in the belief that the materials may be useful in physiological studies when it is found desirable to use the growth-promoting material in a concentrated form.

## CLINICAL CALORIMETRY

### XLVIII. NITROGEN EQUILIBRIUM WITH A LOW PROTEIN DIET

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#### INTRODUCTION

In the course of studies of the nitrogen balance in various conditions we were able to follow a patient with diabetes mellitus for a period of 6 months. It was possible to maintain him in nitrogen equilibrium with an intake of only 3.2 gm. of nitrogen per day (1).

A review of the work in relation to minimal nitrogen studies has been compiled recently at the Missouri Agricultural Station by Brody and his coworkers (2). Total nitrogen excretion in the urine, of less than 2.0 gm. per day, has been reported by four observers (3-6). The lowest point reached was 1.58 gm. of nitrogen per 24 hours in a patient studied by Smith (5). As the intake of nitrogen in these minimal studies was very low, the patients were not in nitrogen equilibrium. Petrén (7) also found low values for the urinary nitrogen ranging from 1.3 to 3.0 gm. per day in diabetic patients who were receiving between 3 and 4 gm. of nitrogen in their food, but the specimens were collected under average ward conditions so that the possibility of the loss of a small amount of urine was not excluded.

#### Case History

The patient, S. A., was first admitted to Bellevue Hospital, September 2, 1924. He was a Hebrew, born in Austria 32 years ago. His past history and his family history were not related to his present condition, which dated from January, 1924, when he had a series of furuncles on his skin associated with polydypsia, polyuria, and some loss of weight. During the interval of 9 months since the onset, he received 10 units of insulin daily

with a diet containing 50 gm. of carbohydrate. His physical examination at the time of admission showed a well developed but poorly nourished adult with dry skin, furred dry tongue, and a number of small infected areas in the skin. The examination showed him to be otherwise normal. Under treatment the skin infections cleared and he was discharged with a diet containing 60 gm. of carbohydrate, 60 gm. of protein, and 154 gm. of fat with 10 units of insulin daily. The urine was sugar-free and the blood sugar was 110 mg. per 100 cc. of blood.

In the interval preceding his present admission to the hospital on October 31, 1925, he had not followed his diet carefully and had omitted his insulin. Examination at this time showed a markedly emaciated drowsy individual with a strong acetone odor on his breath. The urine contained large amounts of sugar and acetone bodies. The blood contained 560 mg. of glucose and showed a carbon dioxide-combining power of 39 volumes per cent.

At this time he was admitted to the Metabolism Ward of the Russell Sage Institute of Pathology. He responded quickly to the administration of fluids and insulin and at the end of 24 hours his urine was free from acetone bodies and the carbon dioxide-combining power was normal. At the end of 3 days the urinary sugar had disappeared. His weight was 44.6 kilos. He received a diet containing 64 gm. of carbohydrate, 40 gm. of protein, and 132 gm. of fat with 35 units of insulin per day. During the next month he showed a tendency to lose weight and his diet was subsequently increased to 132 gm. of carbohydrate, 50 gm. of protein, and 135 gm. of fat, a total of approximately 2000 calories. This was sufficient to maintain him in nutritional equilibrium as judged by the constant level of weight during the rest of the observation. The details of subsequent changes in the composition of his diet may be observed from Table I. In the course of the study the protein in the diet was reduced in three steps from 50 to 20 gm. per day. In these changes the total calories were kept constant by the addition of fat. He was maintained on this low protein intake for a period of 106 days. The lowest daily urinary nitrogen excretion of 1.78 gm. was noted on the 82nd day. For the last 28 days the average daily nitrogen excretion was 2.4 gm.

After he had received 20 gm. of protein daily for 1 month, the

carbohydrate in the diet was gradually reduced and fat increased each time to maintain the total energy value at this same level, until at the lowest point he received only 30 gm. of carbohydrate a day. As a result of the restriction of the carbohydrate in the diet his insulin requirement was reduced to 20 units per day and acetone bodies appeared in the urine. The details for this period of the study have been presented in a previous communication (8).

At the end of 106 days the protein in the diet was again increased and he was discharged from the ward later with a diet containing 50 gm. of protein, 108 gm. of carbohydrate, and 148 gm. of fat, with 35 units of insulin per day.

Subsequent to the admission when the detailed studies were made he was twice a patient in the general wards with impending coma which responded to the administration of fluids and insulin. At the present time, 5 years after the special studies were made, he is working regularly every day but in order to keep himself in good physical condition he lives at a hospital where his diet and insulin requirements are carefully controlled.

#### *Experimental Results*

The detailed observations are presented by periods in Table I. The content of the diets was calculated from the figures given by Rose (9) and the values were checked by the analysis of duplicate diets from time to time. We found no significant variation in the content of the analyzed diet from the data obtained by calculation. The low protein intake continued for 106 days during Periods 4 to 18 inclusive. The nitrogen in the urine was determined by the Kjeldahl method. The stool nitrogen was obtained for two periods, one of 14 days shortly after starting on the low protein level and the second for the last 11 days of the period. For the remainder of the study the stool nitrogen has been estimated on the basis of these two periods and on the findings of Petrén (7) who noted in his extensive studies of the effect of low protein diets in diabetes that the nitrogen in the feces under these conditions varied between 0.5 and 1.0 gm. per day. Our observations were consistent with his conclusions. In the early part of this long period the patient showed a slightly negative nitrogen balance but at the end of the observation he was definitely in nitrogen equilibrium.

During the period of low protein intake the patient's weight varied between 43 and 45 kilos. He showed no striking variation from the average level of 44 kilos over the entire period. An increase in weight of about 2 kilos was noted in the 10 day period

TABLE I  
*Food and Urine Data with Nitrogen Balance*

All data are given in daily averages.

Period No.	Duration of period	Weight	Food intake			N <sub>2</sub> excreted			Balance	Aceto- tone bodies		
			Carbohy- drate	Fat	Nitro- gen	Urine	Feces	Total				
days	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.		
1	7	43.9	132.3	135.1	8.02	6.49	1.00	7.49	+0.53			
2	3	44.1	131.9	138.9	6.42	5.34	1.00	6.34	+0.08			
3	4	44.4	132.3	143.9	4.80	4.56	1.00	5.56	-0.76			
4	8	44.2	132.0	147.7	3.20	3.43	0.75	4.18	-0.98			
5	14	44.4	132.4	148.2	3.20	3.03	0.75*	3.78	-0.58			
6	11	44.3	132.1	148.2	3.22	2.90	0.67	3.57	-0.35			
7	3	43.9	102.4	134.2	2.85	2.95	0.67	3.62	-0.77	0.07		
8	4	44.4	99.8	157.9	3.25	2.76	0.67	3.43	-0.18	0.11		
9	7	43.9	79.5	163.9	3.22	2.82	0.67	3.49	-0.27	0.34		
10	8	44.2	66.1	177.3	3.22	2.84	0.60	3.44	-0.22	0.53		
11	13	43.9	53.1	183.3	3.20	2.96	0.60	3.56	-0.36	1.03		
12	4	44.0	40.0	178.4	3.22	2.69	0.60	3.29	-0.07	1.91		
13	6	43.6	32.3	185.3	3.22	2.89	0.53	3.42	-0.20	3.70		
14	6	44.0	53.0	183.1	3.23	2.40	0.53	2.93	+0.30	0.92		
15	5	44.0	29.9	181.7	3.23	2.75	0.53	3.28	-0.05	2.96		
16	6	43.9	52.9	160.2	3.23	2.41	0.53	2.94	+0.29	1.00		
17	5	43.6	80.1	157.0	3.22	2.28	0.45*	2.73	+0.49	0.12		
18	6	43.9	115.1	148.8	3.20	2.46	0.45*	2.91	+0.29	0.11		
19	4	44.8	119.6	150.3	4.82	2.33	0.75	3.08	+0.74	0.21		
20	3	45.0	105.3	139.4	6.43	2.81	0.75	3.56	+2.87	0.49		
21	6	45.5	104.6	140.9	7.98	3.49	0.75	4.24	+3.74	0.27		

\* Figures obtained by actual analyses of stools. All other figures for fecal nitrogen are estimates.

when the protein in his diet was increased from 20 to 50 gm. At the end of the period of restriction it was interesting to note that in the last period the nitrogen in the urine had increased less than 1 gm. per day, even though the nitrogen in the diet was more than doubled.

Observations of the patient's respiratory metabolism were made from time to time during the period when he was receiving the small amount of protein. His basal metabolic rate was approximately between 85 and 90 per cent of the Aub-Du Bois standards. During Periods 8 to 17 when the carbohydrate and the fat in the diet were shifted isodynamically there was a slight elevation in his basal heat output which occurred when the carbohydrate was at the lower levels. This increase was possibly associated with the ketosis that was present at those times.

#### DISCUSSION

As may be seen from the experimental data presented, we found that during the early part of the time when he was receiving only 20 gm. of protein in his diet that he was in negative nitrogen balance of approximately 1 gm. per day. With the continued slight depletion in his nitrogen reserve, we found him to be definitely in equilibrium for the last month of this prolonged period. It appeared from the fact that he retained much more nitrogen in Period 21 than in Period 1 on an intake in both periods of approximately 8 gm. per day, that the prolonged low protein diet did actually deplete his nitrogen reserve. We detected no evidence that this depletion caused any disturbance in this subject. It required a period of at least 3 or 4 days following any change in his diet before its effect on the metabolism was fully evident. This was definitely noted in the periods preceding the lowest protein level and also in the three concluding periods of the study.

The low level reached in the excretion of nitrogen in the urine, 1.78 gm. per day, has been noted as far as we can determine from the literature in only two other carefully controlled subjects: first, in the patient reported by Smith (5) where the total excretion was 1.58 gm. on the 24th day of a diet which contained less than 0.5 gm. of nitrogen, and secondly, when Deuel (6) reduced his own nitrogen excretion in the urine to 1.75 gm. per day after a prolonged depletion of his nitrogen reserve. Neither of these subjects was in nitrogen equilibrium. When his nitrogen output was calculated on the basis of weight our subject excreted 0.0405 gm. per kilo per day. A number of other subjects have shown lower levels when calculated on this basis.

During the time of the gradual reduction of carbohydrate with

the addition of fat to provide the same amount of energy in the diet, we noted a gradual increase of acetone bodies in the urine. They first appeared when the ratio of fatty acids to available glucose as determined by the Woodyatt formula for the foodstuffs utilized reach 1.5. There was a marked increase in the elimination of acetone bodies when the ratio was 2.0 or above.

The substitution of fat for carbohydrate in the diet did not lead to any perceptible increase in the urinary nitrogen excretion. It should be pointed out that the results of the maximum carbohydrate restriction were observed only in the latter portion of the time when he was receiving the low protein diet. It appears that under these conditions fat may be as good a sparing of protein as carbohydrate. This observation has been emphasized before by Petrén (7). It is possible, however, that a similar restriction of carbohydrate in a person who is receiving adequate amounts of protein in his diet may result in some increase in the protein metabolism as determined from the nitrogen in the urine. This fact may have in part been responsible for the earlier conclusion that carbohydrate spared protein better than fat.

Observation of the weight curve revealed that our subject received in his food sufficient energy to provide for his bodily needs. The diet contained 2000 calories per day during the major part of his stay in the ward. His basal metabolism as determined in the calorimeter was approximately 1200 calories per 24 hours. If we allow for a 10 per cent loss of energy in the food-stuffs which are not absorbed from the intestinal tract, our data then indicate that he used daily about 1800 calories. This represents a maintenance diet of approximately 50 per cent above his basal heat production. It is often stated that a patient in bed needs a diet containing only 20 per cent more than his basal heat production to provide him with sufficient energy to maintain him in nutritional equilibrium. Our subject was in bed during the entire period of the observation but he was active at times, sitting up, reading, and talking to the other patients. These facts may explain the differences in the extra energy required to maintain him in nutritional equilibrium.

The basal metabolism during the period when he was receiving the low protein diet showed a low level of approximately 50 calories per hour. In the observations made during the periods when he

was excreting a large amount of acetone bodies, we found that his respiratory quotient varied between 0.74 and 0.76, while in the periods when he was utilizing larger amounts of carbohydrate, the respiratory quotient was between 0.78 and 0.81.

#### SUMMARY

1. A patient with diabetes mellitus was observed for 106 days on a diet containing 20 gm. of protein per day.
2. Nitrogen equilibrium was established with this low protein intake during the last month of this period.
3. A low level of nitrogen excretion in the urine of 1.78 gm. per day was reached. This represents one of the lowest levels of urinary nitrogen excretion on record.
4. A restriction of the carbohydrate in the diet to 30 gm. per day resulted in the production of 3 to 4 gm. of acetone bodies, but no increase in the breakdown of protein in the body was noted.
5. Weight equilibrium was maintained throughout the entire period and as far as we could observe no ill effects from the prolonged low level of protein in the diet occurred.
6. Nutritional equilibrium was maintained with a diet in which the available energy value was about 50 per cent above the average basal heat output of the patient.

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## A MODIFICATION OF THE VAN SLYKE NITROGEN DISTRIBUTION METHOD

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In 1911 Van Slyke (1) published a method for the analysis of hydrolyzed proteins, which gives information concerning the amount of ammonia, humin, arginine, lysine, histidine, and cystine, and of the total and amino nitrogen fractions of the monoamino acids in the filtrate. 2 or 3 gm. were required for an analysis.

In the author's study of the proteins in nephritic blood and certain other body fluids, it was sometimes difficult to obtain more than 0.5 to 1 gm. of protein. Narayana and Sreenivasaya (2) made a modification of the Van Slyke method for nitrogen distribution which requires only 0.1 gm. of protein, but this did not seem altogether adequate for the work at hand. Therefore the task of developing a method which would require only 0.5 gm. of protein, and still retain the accuracy of the original Van Slyke method, was undertaken. The aims of the method are (a) to eliminate as many transfers of material as is possible, (b) to substitute centrifugation for all filtrations, as the former is more rapid and gives less chance for error, and (c) to modify certain pieces of the apparatus so as to permit the analysis of smaller quantities of nitrogen. These modifications have developed into a method which requires less than one-half the previous time for an analysis, uses one-fifth the amount of sample, and still retains the accuracy of the original method.

### *Modified Method*

0.5 gm. of the protein is hydrolyzed by boiling for 36 hours with 7 cc. of 25 per cent hydrochloric acid in a 250 cc. distilling flask.

A test-tube (closed with a 2-hole rubber stopper carrying two glass tubes, one of which extends to the bottom) inserted in the neck of the flask serves as a reflux condenser. After the digestion the flask containing the protein hydrolysate is placed in a water bath at 60° and vacuum-distilled until a paste remains. A few cc. of water are added and the process repeated so as to remove as much hydrochloric acid as possible. The hydrolysate is quantitatively transferred<sup>1</sup> to a 50 cc. volumetric flask and two 5 cc. aliquots are removed for the total nitrogen determination by the author's micro-Kjeldahl method (3).

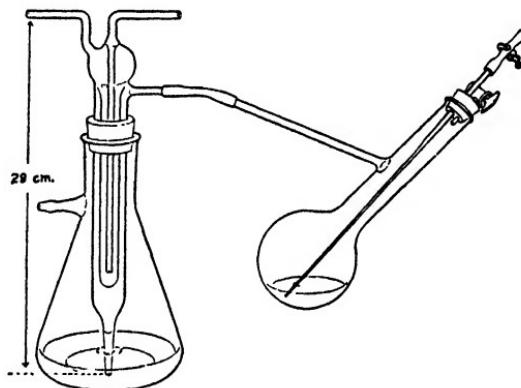


FIG. 1. Ammonia nitrogen distillation apparatus

#### *Ammonia Nitrogen*

The hydrolysate in the 50 cc. volumetric flask is transferred with rinsing into the original distillation flask. The apparatus used for the ammonia nitrogen determination is shown in Fig. 1.<sup>2</sup> The 500 cc. suction flask contains 10 cc. of 0.1 N acid and 100 cc. of distilled water with 3 or 4 drops of methyl red as an indicator. 1 cc. of butyl alcohol and 0.5 gm. of finely pulverized calcium oxide (4) are added to the hydrolysate and the distillation flask

<sup>1</sup> This transfer may be avoided by passing directly to the ammonia nitrogen determination, in which case total nitrogen is determined on another sample of the protein.

<sup>2</sup> If the flask is not placed at the angle shown, bubbles will pass up into the neck and thus over into the standard acid. In this position they always break before entering the neck of the flask and give no trouble.

immediately closed and placed in a water bath heated to 45–50°. Rapid ebullition during the first few seconds of evacuation may be avoided by pinching the tubing connecting the flask and condenser. The distillation is continued until 10 cc. of fluid remain. The excess acid in the suction flask is titrated with 0.02 N sodium hydroxide.

#### *Humin Nitrogen*

The contents of the distillation flask, from which the ammonia has been removed, are transferred to a 50 cc. centrifuge tube. The flask is rinsed with small portions of water, as the total volume must not exceed 25 cc. The humin material is separated by centrifugation and washed three times with 5 cc. portions of water by centrifugation.

The washed precipitate is subjected to Kjeldahl digestion as for total nitrogen, except that it is carried out in the distillation flask which still contains some adhering humin.

#### *Phosphotungstate Precipitation and Separation*

The mother liquor and washings from the humin precipitate are collected in a 100 cc. centrifuge tube. 5 cc. of concentrated hydrochloric acid are added, followed *immediately* (5) by 2.5 gm. of phospho-24-tungstic acid dissolved in a few cc. of hot water. The volume is adjusted to 50 cc. The tube is placed in a hot water bath for 1 hour. It is allowed to cool, stoppered, and placed in an ice box at 0° for 48 to 72 hours. Several hours before the final separation is made the tube is shaken in such a manner as to cause floating particles and those adhering to the side to sink. It is then centrifuged and returned to the ice box.

For the final separation and washing of the phosphotungstate precipitate the tube is again centrifuged, care being taken to see that floating or adhering particles sink to the bottom. The liquid is gently decanted into a 100 cc. volumetric flask and the centrifuge tube chilled in ice water. The precipitate is washed three times with 4 cc. portions of cold acid mixture (10 cc. of concentrated hydrochloric acid and 2.5 gm. of phosphotungstic acid per 100 cc. of solution). Each time the precipitate is broken up with a stirring rod. After centrifugation the wash liquid is added to the original solution in the 100 cc. flask. The solution is then

neutralized by adding 1:1 sodium hydroxide solution until a white precipitate begins to form. This is *immediately* dissolved with glacial acetic acid. After room temperature is reached, the solution is diluted to volume.

The phosphotungstate precipitate remaining in the centrifuge tube is dissolved by suspending it in 5 cc. of water and adding 4 or 5 cc. of N sodium hydroxide. Any undissolved material is allowed to settle and the clear liquid is decanted into a 50 cc. volumetric flask. A few cc. more of water and N sodium hydroxide are again added to the undissolved precipitate remaining in the centrifuge tube and the above process repeated. Sometimes a third addition of water and sodium hydroxide is required for complete solution.

After the solution of the phosphotungstate precipitate has thus been transferred to the 50 cc. flask, 1 drop to 0.1 per cent solution of phenolphthalein is added. (1 drop in 50 cc. of alkaline water gives a pink color which is just distinguishable against a white background. 5 cc. of this water solution diluted to 25 cc. do not give a perceptible color, which would interfere with the cystine determination.) The solution is adjusted to a pink color and 1 drop of glacial acetic acid added. It is then diluted to volume.

#### *Analysis of Phosphotungstate Precipitate and Filtrate*

*Nitrogen*—Nitrogen is determined in duplicate upon 5 cc. portions of the basic fraction and 25 cc. of the filtrate. The determinations are carried out by micro-Kjeldahl analysis (3). Because of the presence of phosphotungstic acid, the digestion is continued 3 hours after the solution has become clear. For the digestion of the filtrate fraction 6 cc. of the concentrated sulfuric acid are used instead of 4 cc.

*Amino Nitrogen*—This is determined on 2 cc. portions of the basic and the filtrate fractions with Van Slyke's micro amino nitrogen apparatus (6).

*Arginine Nitrogen*—The apparatus used for the arginine determination is a modification of that used by Holm (7) (Fig. 2). 10 cc. of the basic fraction solution are placed in a 300 cc. Florence flask and 10 cc. of 1:1 sodium hydroxide solution added. Three glass beads are added to prevent bumping. The large test-tube contains 10 cc. of 0.02 N acid. Water is allowed to pass

through the condenser and the contents of the flask are boiled gently over a micro burner for 6 hours. The apparatus is allowed to cool and the condenser is drained. 100 cc. of water and a few grains of sand are added to the Florence flask through the funnel and stop-cock. Ice and water in a beaker are placed around the test-tube which contains the standard acid. The last traces of the ammonia are then distilled over into the acid. The titration is made with 0.02 N alkali with the micro burette.

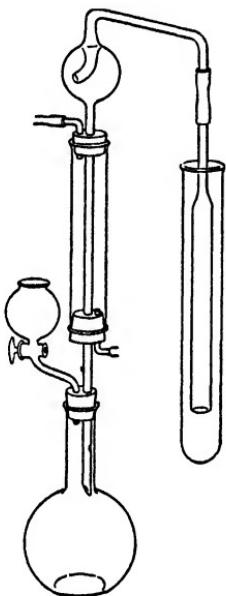


FIG. 2. Arginine nitrogen apparatus

*Cystine Nitrogen*—A modification of the colorimetric method of Folin and Marenzi (8) is used for the determination of cystine nitrogen. 5 cc. of the solution obtained from the phosphotungstate precipitate are placed in a 25 cc. volumetric flask and 2 drops of 50 per cent sulfuric acid added, thus producing an acidity similar to that of the standard solution. The standard cystine solution is a solution of N sulfuric acid containing 17.16 mg. of cystine per 100 cc.; thus 1 cc. contains 0.02 mg. of cystine nitrogen. The necessary amount of the standard solution (usually 2 to 5 cc.) is added to a 25 cc. volumetric flask. The volume of

liquid in the standard flask, during the color development, should be equal to that in the unknown flasks. 0.5 cc. of freshly prepared 20 per cent sodium sulfite solution is added to the standard and unknown. After this has stood for 1 minute, 4.5 cc. of a 20 per cent solution of sodium carbonate, 0.5 cc. of 20 per cent lithium sulfate, and 2 cc. of Folin's new molybdate-free uric acid reagent are added. After standing for 3 to 4 minutes, the solutions are diluted to volume with a freshly prepared 3 per cent solution of sodium sulfite and compared in a colorimeter. Cystine has been quantitatively recovered by this method from known solutions containing the amounts of phosphotungstic acid and phenolphthalein present in the unknowns.

The cystine which is not precipitated and remains in the filtrate may also be determined by placing 10 cc. of the filtrate solution in a 50 cc. centrifuge tube graduated at 25 cc. The reagents are added as for the basic fraction, except that the solution is immediately diluted to 25 cc. and centrifuged before comparison with the standard. This is necessary, as a precipitate of calcium carbonate forms when the sodium carbonate is added. The same standard serves for both the basic fraction and the filtrate, as only 40 to 50 per cent of cystine is precipitated with phosphotungstic acid after it has been boiled with hydrochloric acid.

*Histidine*—This is determined by the colorimetric method of Koessler and Hanke (9). An aliquot of the basic fraction is diluted 1:10 and 1 cc. of this solution is used for the determination.

1.5 cc. of sulfanilic acid solution (0.9 gm. of sulfanilic acid dissolved in 9 cc. of 37 per cent hydrochloric acid and diluted to 100 cc.) are placed in a 50 cc. flask immersed in ice water and 1.5 cc. of 5 per cent sodium nitrite solution added. After 5 minutes 6 cc. more of the nitrite solution are added. In 5 minutes the solution is diluted to 50 cc., and is ready for use after 15 minutes.

7.5 cc. of 1.1 per cent pure anhydrous sodium carbonate solution are placed in a large test-tube and 0.5 cc. of water added. 3 cc. of the above reagent (*p*-diazobenzene sulfonic acid) are added, the tube shaken, and in exactly 1 minute 1 cc. of the unknown solution is added and thoroughly mixed. After 6 minutes the solution is compared in a colorimeter with an artificial standard. An artificial standard is used because the time element in adding

the histidine solution to the reagents is very important and it is difficult to handle two solutions at once. The artificial solution is prepared by diluting 0.82 cc. of 0.5 per cent Congo red solution with 300 cc. of water. To this is added 0.9 cc. of 0.1 per cent methyl orange solution, and the volume is brought to 500 cc. This artificial standard should give a color equivalent to 0.009 mg. of histidine nitrogen in 12 cc. but should be rechecked against

TABLE I  
*Accuracy of Modified Nitrogen Distribution Method*

The figures are expressed as percentage of total nitrogen.

	Variations in duplicate analyses				Casein analysis	
	Modified method		Van Slyke's limits*		Original method†	Modified method
	Maximum	Average	Maximum	Average		
Amide N.....	0.50	0.19	0.37	0.12	10.27	10.51
Humin “.....	0.40	0.11	0.39	0.20	1.28	2.03
Cystine N						
Precipitate.....	0.92	0.21	0.11	0.05	0.20	0.24
Filtrate.....	0.82	0.23				
Total.....	0.23	0.07				
Arginine N.....	0.66	0.30	1.27	0.73	7.41	7.67
Histidine “.....	1.11	0.53	2.14 (.93)	0.79	6.21	4.49
Lysine N.....	0.78	0.21	1.21	0.61	10.30	10.35
Filtrate N						
Amino.....	1.54	0.51	1.60 (.60)	0.63	55.81	56.34
Non-amino.....	0.93	0.29	1.20	0.68	7.13	8.96
Total nitrogen recovered.....					98.61	100.59

\* Cf. (1) p. 32.

† Van Slyke, D. D., *J. Biol. Chem.*, 16, 538 (1913-14).

a standard histidine solution whenever a new set of reagents is used, owing to variations in the purity of the reagents and hence the color produced. A convenient stock standard for this purpose contains 5.43 mg. of histidine dichloride (1 mg. of nitrogen) in 1 cc. of 0.05 N hydrochloric acid. 1 cc. of the stock standard and 0.5 gm. of phosphotungstic acid diluted to 100 cc. serve as a working standard and contain 0.01 mg. of histidine nitrogen per cc.

Sometimes a perfect color match is not obtained, but no dif-

ficulty is experienced in reading if the special blue filter for the hemoglobinometer of Bausch and Lomb (catalogue No. 3610) is used since the color absorption of this solution is in the short wave-lengths.

Thymol must not be used as a preservative, as it interferes with the histidine determination.

### *Results*

Duplicate determinations have been made on samples of casein, gliadin, edestin, albumin, and pseudoglobulin from blood, three albumin samples from urine, and samples of globulin obtained from thyroid glands. The average and maximum variations obtained in duplicate analyses on these proteins are given in Table I. Also the average of the results from the two analyses upon casein (prepared by the Harris Laboratories, Tuckahoe, New York) are compared with those given by Van Slyke (10) for the original method. The humin figure is 0.75 per cent higher by the modified method, but this is probably due to traces of lipids (for the casein sample, as is given in the manufacturer's analysis, contains 0.2 per cent ether-soluble material). The presence of lipids in proteins increases the humin fraction, as is shown by the author's unpublished work on serum proteins and also by the work of Hauge (11).

### SUMMARY

By adoption of micro-Kjeldahl instead of macro-Kjeldahl analyses the Van Slyke nitrogen distribution method has been modified so that only 0.5 gm. of protein is required for an analysis.

The washing of the humin and phosphotungstate precipitates has been simplified by using centrifugation.

Cystine is determined by the Folin-Marenzi colorimetric method.

Histidine is determined directly by the colorimetric method of Koessler and Hanke in place of being calculated.

The author is indebted to Dr. D. D. Van Slyke for suggesting the colorimetric histidine method.

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# THE FRACTIONAL PRECIPITATION OF SERUM GLOBULIN AT DIFFERENT HYDROGEN ION ACTIVITIES. EXPERI- MENTS WITH GLOBULIN OBTAINED FROM NORMAL AND IMMUNE (ANTIPNEUMOCOCCUS) HORSE SERUM

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The problem as to whether or not the globulin fractions obtained from blood serum are chemical entities has been the subject of much discussion recently. Svedberg (1), basing his views on particle (molecular) weight determination, came to the conclusion that there is only one serum globulin, the molecular weight of which he determined to be 103,800. Sørensen (2) concluded, however, from solubility measurements that the globulin fractions, like many other proteins, consisted of two components, each of which was of protein nature. He assumed that one of these components was the water-soluble globulin (pseudoglobulin) and that the other was insoluble in distilled water (euglobulin). The peculiar fact that comparatively less globulin was precipitated from concentrated than from dilute solutions under otherwise identical conditions was attributed by Ostwald (3) to the colloidal character of the globulin. He pointed out that the dispersion of colloids in general depends on the amount of solid phase present, for the amount of peptizing agent as a rule is increased in proportion to the total amount of substance used in a solubility determination. This explanation also involved the assumption that the globulin consisted of at least two components.

In the course of an attempt to purify serum globulin by means of electrodialysis (4) it was found that this procedure, even when repeatedly applied to the same substance, always yielded two fractions: one which was insoluble at its isoelectric zone and

another, which was soluble at any hydrogen ion activity in distilled water. If the insoluble fraction was redissolved in a salt solution and reelectrodialyzed, a protein was obtained, which did not yield a clear solution in physiological salt solution, but only a milky suspension. Since the electrodialysis was conducted very carefully so that it did not produce any denaturation when it was applied to native serum, it was concluded that the insolubility of the electrodialyzed globulin was not due to denaturation of protein. If after electrodialysis the supernatant solution was reprecipitated by salting out, and reelectrodialyzed, it yielded more of the protein insoluble in distilled water. Most of the serums used in these experiments were extracted according to Hardy and Gardiner (5), hence the peculiar behavior observed could not be attributed to the presence of free lipoids (*cf.* also Sørensen (6)). Thus both fractions obtained by electrodialysis (and similar ones obtained by simple dialysis) were apparently mixtures. It was assumed that the globulin is associated in its native state with some other substance which helps to keep it in solution. The nature of this agent could not be determined. Several attempts to make the insoluble globulin soluble by addition of some protective colloid, heparin among others, failed to produce the desired effect (7). In order to obtain some information about its complexity, it was also attempted to fractionate the globulin by precipitation at different hydrogen ion activities, but without success. This lack of success was evidently due to the fact that we used a globulin which had been precipitated several times and was thus already devoid of the greater part of one of its "components." Besides this, we selected for the fractionation hydrogen ion activities too close to each other.

Two fractions of serum globulin were obtained recently by precipitation at different hydrogen ion activities by Felton (8) from antipneumococcus horse serum. One of these fractions was precipitated at about pH 5 and was less soluble in dilute salt solutions than the other which was precipitated at pH 6.8. These two fractions were considered to be two different proteins. This view was supported by the fact that the biological activity of these fractions was different. The one which was precipitated at a lower pH (acid fraction) contained little or no antibody, the other (neutral fraction) carried the specific immune properties of

the immune serum. The chemical and physical properties of these two proteins have not been studied extensively in spite of the fact that it would seem that at least the antibody-carrying protein is a hitherto unknown sort of serum globulin.

Owing to lack of information concerning the processes which take place when the antipneumococcus serum is treated in the manner mentioned above, it is still questionable whether or not the proteins obtained are present in a free state in the serum. In the light of the experiments of Svedberg and Sjögren (9), it becomes conceivable that proteins can be decomposed, even at hydrogen ion activities which are comparatively near to neutrality, in a relatively short time. Thus the globulin fractions obtained at different hydrogen ion activities may also be considered as dissociation products of the native globulin. The experiments reported in this paper will deal with this problem and also with the comparison of the fractionation of normal serum globulin and immune serum globulin.

In the first group of these experiments attempts were made to demonstrate in the serum the presence of the two proteins, which have different precipitation optiums. The amount of protein, which is precipitable at different hydrogen ion activities from normal serum and from immune serum, was therefore determined.

In the second group of experiments the two fractions of the globulin were prepared from normal serum and from immune serum and their precipitability at different hydrogen ion activities studied. Some information was obtained regarding the reversibility of the separation by precipitating the mixture of the two fractions at different hydrogen ion activities and comparing the results with those obtained when single fractions were precipitated.

Finally in order to characterize the fractions further, their acid- and base-binding capacities and their nitrogen contents were determined.

*Precipitation of Globulin by Dilution of Serum at Different Hydrogen Ion Activities.*—The hydrogen ion activities of samples of normal serum and immune serum were adjusted to various values. The samples were then diluted to 15 times their original volume and kept for 2 days in the ice box. The amount of protein precipitated by this procedure was determined. (For details

see p. 357.) Curves were obtained representing the variation of the amount of protein precipitated at different pH. If two proteins, precipitable at different hydrogen ion activities, were present in the serum in a free state, the curves should have two maximums or a flat maximum between these points if the precipitation zones are wide and overlap. In the case of antipneumococcus immune serum one would expect, according to Felton, one maximum at about pH 5.2 and another at about pH 6.8. The antipneumococcus serum should differ in this respect from the normal horse serum, which is supposed to have only one of these proteins and therefore only one precipitation optimum. Our experiments showed, however, that there is no qualitative difference between

TABLE I  
*Precipitation of Antipneumococcus Horse Serum (No. 202) at Different pH and 1:15 Dilution*

Fraction No.	Part of fraction	Electrolyte (estimated)	0.1 N HCl added to 100 cc. serum	pH	N per 100 cc. original serum
		m.-eq. per l.	cc.		mg.
1	Supernatant	0.017	16.0	6.85	1152
1	Precipitate	0.017	16.0	6.85	124
2	Supernatant	0.015	38.0	5.90	1077
2	Precipitate	0.015	38.0	5.90	211
3	Supernatant	0.013	42.0	5.65	945
3	Precipitate	0.013	42.0	5.65	260
4	Supernatant	0.012	50.0	5.10	1236
4	Precipitate	0.012	50.0	5.10	61

normal serum and immune serum in this respect. Both have only one precipitation zone with an optimum at pH 5.5 to 6.0 (*cf.* Tables I to III).

*Precipitation of Single Fractions and Their Mixtures*—As already mentioned, the fact that only one precipitation optimum is observed, when globulin is precipitated from serum at different hydrogen ion activities, does not exclude the possibility that there are two globulin-like proteins present in the serum, which possess different electrochemical properties. Indeed, it was found that if the precipitation of the proteins is carried out in two steps, namely if the protein which is precipitated at about pH 5.2 is precipitated first and then the other fraction is obtained by further dilution and

neutralization to pH 6.8, two proteins are obtained with distinctly different precipitation zones from immune serum as well

TABLE II  
*Precipitation of Horse Serum (No. 201\*) at Different pH and 1:15 Dilution*

Fraction No.	Part of fraction	Electrolyte (estimated)	0.1 N HCl added to 100 cc. serum	pH†	N per 100 cc. original serum
		m.-eq. per l.	cc.		mg.
1	Supernatant	0.012	15.0	6.9	1265
1	Precipitate	0.012	15.0	6.9	24
2	Supernatant	0.009	22.0	6.5	1196
2	Precipitate	0.009	22.0	6.5	82
3	Supernatant	0.011	26.0	6.1	1148
3	Precipitate	0.011	26.0	6.1	114
4	Supernatant	0.011	34.0	5.7	1195
4	Precipitate	0.011	34.0	5.7	81
5	Supernatant	0.011	38.0	5.4	1193
5	Precipitate	0.011	38.0	5.4	75
6	Supernatant	0.011	50.0	5.1	1252
6	Precipitate	0.011	50.0	5.1	36

\* The horse was not immunized during the year preceding the bleeding.

† Determined colorimetrically.

TABLE III  
*Precipitation of Normal Horse Serum at Different pH and 1:15 Dilution*

Fraction No.	Part of fraction	Electrolyte (estimated)	0.1 N HCl added to 100 cc. serum	pH*	N per 100 cc. original serum
		m.-eq. per l.	cc.		mg.
1	Supernatant	0.012	11.7	6.8	1035
1	Precipitate	0.012	11.7	6.8	41
2	Supernatant	0.012	31.7	6.2	1019
2	Precipitate	0.012	31.7	6.2	63
3	Supernatant	0.011	40.0	5.9	1002
3	Precipitate	0.011	40.0	5.9	63
4	Supernatant	0.011	44.7	5.5	1013
4	Precipitate	0.011	44.7	5.5	77
5	Supernatant	0.012	50.0	5.2	1030
5	Precipitate	0.012	50.0	5.2	35

\* Determined colorimetrically.

as from normal serum. (The process of fractional precipitation is described on p. 356; the amount of nitrogen-containing sub-

stance precipitated from these fractions at different hydrogen ion activities is given in Tables IV and V.)

The question arises now whether or not these two fractions were present in an uncombined state in the serum. In order to decide this the precipitation of the mixture of the two fractions was studied.

TABLE IV  
*Precipitation of Fractions Obtained from Antipseudomycococcus Horse Serum*  
Dilution = 1:10; equivalent of electrolyte per liter = 0.0161.

Fraction	Part of fraction	0.1 N NaOH or HCl added	pH	N per 100 cc. undiluted solution	Nitro- gen	N per 100 cc. undiluted solution (calculated)	N (calculated)
		cc.		mg.	per cent	mg.	per cent
1, neutral	Supernatant	0.50 HCl	6.76	101	10.0		
	Precipitate	0.50 "	6.76	902	90.0		
2, neutral	Supernatant	1.50 "	6.01	610	57.5		
	Precipitate	1.50 "	6.01	454	42.5		
3, neutral	Supernatant	3.00 "	5.49	1050	99.6		
	Precipitate	3.00 "	5.49	5	0.4		
1, acid	Supernatant	0.50 NaOH	6.86	160	100		
	Precipitate	0.50 "	6.86	Trace			
2, acid	Supernatant	0.50 HCl	6.06	77	48.7		
	Precipitate	0.50 "	6.06	80	51.3		
3, acid	Supernatant	1.00 "	5.43	28	17.6		
	Precipitate	1.00 "	5.43	131	82.4		
1, mixture	Supernatant	0.00	6.83	35	5.8	130	22.4
	Precipitate	0.00	6.83	572	94.2	451	77.6
2, mixture	Supernatant	1.00 HCl	5.95	127	19.7	338	55.8
	Precipitate	1.00 "	5.95	511	80.3	267	44.2
3, mixture	Supernatant	2.00 "	5.36	201	32.5	539	89.3
	Precipitate	2.00 "	5.36	418	67.5	65	10.7

From the values obtained, when solutions of a single fraction were precipitated, the values can be calculated which should be obtained for mixtures of fractions, provided that these would behave like ideal mixtures, that is, the amount of protein precipitated at any pH from a mixture would be equal to the sum of protein precipitated from individual solutions under otherwise identical conditions. These calculated values show, as it appears

from Tables IV to VI, only one precipitation optimum. In other words the precipitation curves of the two fractions overlap in such a way that the mixtures possess only one precipitation optimum. Thus it remains possible that the fractions are preformed in the serum. But in this case it is also to be expected that the values calculated for an ideal mixture should be identical within the

TABLE V

*Precipitation of Fractions Obtained from Antipneumococcus Horse Serum*  
Dilution = 1:5; equivalent of electrolyte per liter = 0.0370.

Fraction	Part of fraction	0.1 N HCl added	pH	N per 100 cc. undi- luted solution	Nitro- gen	N per 100 cc. undi- luted solution (calcu- lated)	N (calcu- lated)
		cc.		mg.	per cent	mg.	per cent
1, neutral	Supernatant	0.20	6.76	94	22.9		
	Precipitate	0.20	6.76	316	77.1		
2, neutral	Supernatant	0.50	6.18	143	34.5		
	Precipitate	0.50	6.18	271	65.5		
3, neutral	Supernatant	1.20	5.39	411	98.6		
	Precipitate	1.20	5.39	6	1.4		
1, acid	Supernatant	0.00	6.66	168	78.6		
	Precipitate	0.00	6.66	63	29.4		
2, acid	Supernatant	0.50	6.15	108	48.2		
	Precipitate	0.50	6.15	117	51.8		
3, acid	Supernatant	0.85	5.45	56	25.3		
	Precipitate	0.85	5.45	166	74.7		
1, mixture	Supernatant	0.00	6.70	68	21.7	131	42.2
	Precipitate	0.00	6.70	243	78.2	179	57.8
2, mixture	Supernatant	0.50	6.12	58	18.4	125	39.2
	Precipitate	0.50	6.12	257	81.6	194	60.8
3, mixture	Supernatant	1.00	5.38	147	46.6	233	72.9
	Precipitate	1.00	5.38	168	53.4	86	27.1

experimental error with the experimental values obtained with mixtures. This was, however, not the case. The experimental values (Tables IV to VI) were as a rule much higher than the calculated values, showing that the fractions combine in some way. It must be pointed out that the chances for the occlusion of some soluble protein by the precipitate were the same in the solution of the individual fractions as in the mixture, for the concen-

tration of the mixture was the mean of the concentrations of the individual fractions.

The separation of the two fractions from normal serum was not as complete as from immune serum. The precipitation optimum of both fractions was still about pH 5.5 to 6.0, but one of the fractions was more completely precipitated at pH 5.1 than at pH 6.0,

TABLE VI  
*Precipitation of Fractions Obtained from Antipseudomycoccus Horse Serum*  
Dilution = 1:10; equivalent of electrolyte per liter = 0.0630.

Fraction	Part of fraction	0.1 N NaOH or HCl added	pH	N per 100 cc. undiluted solution	Nitro- gen	N per 100 cc. undiluted solution (calculated)	N (calculated)
		cc.		mg.	per cent	mg.	per cent
1, neutral	Supernatant	0.20 NaOH	6.73	509	59.7		
	Precipitate	0.20 "	6.73	343	40.3		
2, neutral	Supernatant	0.10 HCl	6.02	507	60.4		
	Precipitate	0.10 "	6.02	332	39.6		
3, neutral	Supernatant	1.00 "	5.04	756	87.7		
	Precipitate	1.00 "	5.04	106	12.3		
1, acid	Supernatant	0.10 NaOH	6.78	134	70.8		
	Precipitate	0.10 "	6.78	56	29.2		
2, acid	Supernatant	0.20 HCl	5.96	106	56.2		
	Precipitate	0.20 "	5.96	83	43.8		
3, acid	Supernatant	0.50 "	5.03	71	37.4		
	Precipitate	0.50 "	5.03	119	62.6		
1, mixture	Supernatant	0.10 NaOH	6.64	291	56.8	322	61.8
	Precipitate	0.10 "	6.64	222	43.2	199	38.2
2, mixture	Supernatant	0.15 HCl	5.93	321	59.7	307	59.8
	Precipitate	0.15 "	5.93	217	40.3	207	40.2
3, mixture	Supernatant	0.60 "	5.06	376	73.7	413	79.1
	Precipitate	0.60 "	5.06	134	26.3	112	20.9

the other was more completely precipitated at pH 6.7 than at pH 5.1. The mixture precipitated in an additive way at higher hydrogen ion activities. The proteins did not precipitate in an additive way at pH 6.7 and 6.2 (Table VII).

*Some Physical and Chemical Characteristics of the Components of Globulin*—Besides the determination of the precipitation zone

by which the fractions described are defined, their nitrogen contents and their acid- and base-binding capacities were determined. The nitrogen content was determined for three preparations with the more acid precipitation zone. Two of these were prepared from antipneumococcus and one from normal horse serum. The values are given in Table VIII. In globulins, which he designates

TABLE VII  
*Precipitation of Fractions Obtained from Normal Horse Serum at 1:10 Dilution*

Fraction	Part of fraction	0.1 N NaOH or HCl added	Electrolyte	pH	N per 100 cc. undiluted solution	Nitrogen	N per 100 cc. undiluted solution (calculated)	N (calculated)
		cc.	eq. per l.		mg.	per cent	mg.	per cent
1, neutral	Supernatant	0.14 NaOH	0.0155	6.80	202	43.8		
	Precipitate	0.14 "	0.0155	6.80	259	56.2		
2, neutral	Supernatant	0.00	0.0155	6.34	140	30.2		
	Precipitate	0.00	0.0155	6.34	323	69.8		
3, neutral	Supernatant	0.20 HCl	0.0157	5.37	258	56.2		
	Precipitate	0.20 "	0.0157	5.37	201	43.8		
1, acid	Supernatant	0.50 NaOH	0.0155	6.74	175	71.3		
	Precipitate	0.50 "	0.0155	6.74	70	28.7		
2, acid	Supernatant	0.20 "	0.0155	6.00	107	43.5		
	Precipitate	0.20 "	0.0155	6.00	139	56.5		
3, acid	Supernatant	0.00	0.0155	5.43	55	23.5		
	Precipitate	0.00	0.0155	5.43	179	76.5		
1, mixture	Supernatant	0.25 NaOH	0.0155	6.70	159	44.3	189	53.5
	Precipitate	0.25 "	0.0155	6.70	200	55.7	164	46.5
2, mixture	Supernatant	0.00	0.0155	6.18	135	37.9	124	34.9
	Precipitate	0.00	0.0155	6.18	221	62.1	231	65.1
3, mixture	Supernatant	0.25 HCl	0.0157	5.28	156	45.4	157	45.3
	Precipitate	0.25 "	0.0157	5.28	188	54.6	190	54.7

as sodium carbonate- and alkali-soluble, Lustig (10) found a nitrogen content as low as that of our unextracted fractions.

The acid- and base-binding capacities were determined on electrodialyzed preparations which were dissolved in CO<sub>2</sub>-free physiological salt solution containing no buffering material whatsoever. The water-soluble globulin was used without addition of saline. The titration with NaOH was carried out according to

Willstätter (11), that with HCl according to Linderström-Lang (12). The values obtained are given in Table IX.

Variations which can be found with different preparations may be partly due to differences in their compositions but are mainly

TABLE VIII  
*Nitrogen Content of Globulin Fractions*

Fraction No.	N per gm. protein
4, acid (immune).....	mg. 144.0
2, " " .....	145.5
4, " (normal).....	144.0
Average.....	144.5
2, neutral (immune).....	149.0
4, " (normal).....	150.5
Average.....	149.7
2, acid (immune) before extraction.....	124.0

TABLE IX  
*Acid- and Base-Combining Capacity of Globulin Fractions*

Fraction No.	mm HCl per gm.		mm NaOH per gm.		mm HCl + NaOH per gm.		pH of solution	HCl • NaOH
	Nitro- gen	Pro- tein	Nitro- gen	Pro- tein	Nitro- gen	Pro- tein		
1, acid (immune).....	7.26	1.06	7.98	1.16	15.24	2.32	4.56	0.91
2, " " .....	6.40	0.94	7.00	1.02	13.45	1.96	5.31	0.92
1, " (normal).....	6.26	0.94	6.10	0.91	12.36	1.85		1.04
4, " " .....	5.83	0.85	6.47	0.94	12.30	1.79	5.34	0.90
2, neutral (immune).....	7.10	1.06	4.55	0.68	11.65	1.74	5.82	1.56
4, " (normal).....	6.12	0.92	4.93	0.74	11.05	1.66	6.06	1.25
Water-soluble.....	7.50	1.12	12.40	1.85	19.90	2.97	5.26	0.61

' Corresponds to  $\frac{\text{NH}_3}{\text{COOH}}$  of some authors.

due to the fact that the preparations were probably not exactly at their isoelectric points. The values for the total number of ionizing groups (equivalent of acid + equivalent of alkali per gm. of protein) are much more uniform, because these values are

not affected by the acid or alkali which was not removed by the electrodialysis. It is interesting to note that the total number of ionizing groups was found to be the highest for the water-soluble globulin; it was lower for the acid fraction and lowest for the fraction which can be precipitated near neutrality. These values are of the same order as those obtained by Weber (13) for total globulin and by Lustig for different fractions of serum globulin prepared by salting out and dialysis.

#### DISCUSSION

There are two alternatives by which the experimental results presented can be explained. One has either to assume that there are at least two electrochemically different water-insoluble globulins present in the serum and that they combine with each other at certain hydrogen ion activities, or one has to assume that there is only one globulin, which is built up from two protein components, which have different electrochemical characters. This complex globulin appears to be very unstable on the acid side of the isoelectric point. In this respect it recalls the behavior of hemoglobin.

While most of the recent investigations on globulins have led to the conclusion that they are complexes, built up from different protein components, none of them revealed the nature of the components or the nature of the binding between them. The fractions described in the present paper behave like globulins. Since at certain hydrogen ion activities they have opposite charges, it is possible that they are united by interionic forces. The modern amphionic theory of amphotytes suggests, however, another type of binding between the components. Most of the proteins are ionized at and near their isoelectric point as amphions (Weber (13)). It was suggested by earlier experiments that this holds also for globulins and thus also for the fractions, which are considered in this paper. It is also known that amphotytes show a tendency to associate near their isoelectric point. This has recently been attributed to the attraction between the amphions (14). Now if two amphotytes possess isoelectric points which are near each other, it may be expected that they will associate because of the action of forces similar to those which tend to precipitate proteins at their isoelectric zones. The attraction must be especially great between particles which are flat or longitudinal. Such forms have been suggested by Svedberg and also by Gorter and Grendel (15).

## EXPERIMENTAL

1. *Preparation of Fractions from Normal Serum*—4 liters of normal horse serum were saturated with MgSO<sub>4</sub> (Tested Purity). The mixture was allowed to stand for 2 days at room temperature. The precipitate was filtered and washed with saturated MgSO<sub>4</sub> solution, dissolved by addition of distilled water, and dialyzed against distilled water for 1 day, then filtered and reprecipitated with MgSO<sub>4</sub>. After standing for 2 days it was filtered again. The precipitate was dialyzed against distilled water for 16 days. (The water was changed twice a day, and was saturated with toluene.) The dialysate (470 cc.) was mixed with 62.3 cc. of 0.1 N NaCl and 50 cc. of 0.1 N HCl, yielding a mixture of pH 5.04. The globulin was allowed to precipitate while in the ice box for 2 days, filtered, 72.0 cc. of 0.1 N NaOH added to the filtrate, which was then diluted to 1500 cc. and which had a pH of 6.91. It was allowed to stand for 2 days in the ice box, then centrifuged, and the precipitate washed with a small amount of distilled water, dissolved by addition of 5.0 cc. of 9 per cent NaCl, and made up to 50 cc. with distilled water. The first precipitate obtained at pH 5.04 was dissolved by addition of 2.5 cc. of 9 per cent NaCl and made up to 25 cc. Both fractions were reprecipitated, the acid at pH 5.1, the neutral first at pH 5.1, and the resulting supernatant liquid at pH 6.8.

2. *Preparation of Water-Soluble Globulin*—The supernatant fluid obtained after the precipitation of globulin at 6.91 was electrodialyzed with a low current for 24 hours (0.5 milliamperes per square centimeter maximum current). Towards the end of the electrodialysis the current did not change for several hours. The conductivity of the fluid at the time the electrodialysis was interrupted was approximately  $3 \times 10^{-6}$  reciprocal ohms.

3. *Preparation of Fractions from Immune Serum*—10 liters of serum were dialyzed in ten cellophane bags against running tap water for 2 days. The chlorine content was determined and found to be 0.035 N. The total volume was 14.3 liters. To this was added a mixture of 2400 cc. of 0.1 N HCl and 380 cc. of N NaCl and the whole diluted to 25 liters. The chlorine content was then 0.027 N, and the pH 5.20. It was allowed to precipitate for 2 days. The larger part of the supernatant liquid was siphoned off and the remainder centrifuged. The precipitate was washed

with a small amount of distilled water, dissolved by addition of 40 cc. of 9 per cent NaCl, and made up with distilled water to 400 cc. The supernatant liquid was neutralized with 2600 cc. of 0.1 N NaOH and diluted with twice its volume of distilled water, yielding a mixture of pH 6.90. It was allowed to precipitate for 2 days, then siphoned and centrifuged, the precipitate washed with a small amount of distilled water, and dissolved by addition of 50 cc. of 9 per cent NaCl, and made up with distilled water to 500 cc. Both preparations were reprecipitated. The fraction obtained at pH 5.1 was diluted to 2 liters with distilled water. The neutral precipitate was acidified with 250 cc. of 0.1 N HCl and diluted to 3 liters. The solution then had a pH of 5.20. It was allowed to precipitate for 2 days (ice box). The precipitate was discarded; the supernatant liquid was neutralized with 260 cc. of 0.1 N NaOH and diluted to 9 liters. It was again allowed to precipitate for 2 days. The precipitate was washed with a small amount of distilled water and dissolved by the addition of 30 cc. of 9 per cent NaCl and diluted to 300 cc. The neutral fraction was reprecipitated in a similar way. The values obtained with these preparations are given in Table II.

4. *Precipitation of Globulin from Serum or Protein Solution at Different pH*—10 to 100 cc. samples of protein solution or serum were taken, their pH adjusted by addition of 0.1 N HCl or NaOH, then diluted to 10 times their original volume with distilled water (if not otherwise stated in the tables), the pH determined again, and the globulin allowed to precipitate for 2 days while in the ice box. As a rule once or twice 0.50 cc. samples were taken out for the pH determinations. The precipitate was centrifuged and dissolved by addition of 5 cc. of 9 per cent NaCl and sometimes a few drops of 0.1 N NaOH, and made up to 50 cc. in a volumetric flask, then the nitrogen determined on three samples taken from it. Aliquot parts of the supernatant liquid were taken for nitrogen determinations. The values are given for 100 cc. of undiluted serum or protein solution. Dilutions and samples used for pH determinations were taken into consideration by calculating these values. In the case of experiments with mixtures, solutions were mixed, the precipitation values of which were previously determined and given in the corresponding tables. Equal volumes and not equal amounts of protein were mixed. The acid fraction contained less protein than the neutral fraction.

*5. Analytical Methods*—The protein content was determined by heat precipitation and weighing. The solution was diluted so that its protein content was about 1 per cent and enough NaCl was added to make its concentration also 1 per cent. The solution was made slightly acid (pH 5) and then kept in boiling water for 20 minutes. It was filtered hot through a Jena porous glass crucible, washed with hot distilled water and afterwards with absolute alcohol and dried to constant weight over sulfuric acid in a vacuum. Three to six determinations were made in each case and the average taken. (Average error  $\pm 1$  per cent.) Another method which was used for protein determination was the precipitation with 8 times its volume of absolute alcohol, filtration through a porous glass crucible, extraction in the same crucible with hot alcohol, using the extraction apparatus of Soxhlet-Wiley, drying the residue to constant weight in the vacuum desiccator over sulfuric acid, and weighing. Finally in some cases the solid content of the preparation was also determined, dried to constant weight at 105°, and the amount of NaCl present subtracted. The chloride content was determined by the Volhard method. The nitrogen was determined by the Kjeldahl method. The phosphorus was determined colorimetrically according to Fiske and Subbarow (16). The material was digested with concentrated sulfuric and nitric acids, boiled down several times in order to expel the nitric acid, and an aliquot part used for the determination. The development of the color was complete half an hour after the reagents were mixed. Readings were taken at this time.

*6. Extraction of Preparations*—Extraction was carried out according to Hardy and Gardiner (5). In one instance this extraction was followed by an extraction with hot alcohol in the extraction apparatus of Soxhlet, modified by Wiley.

*7. Electrodialysis of Preparations*—Every preparation which was used for the determination of the acid- and base-binding capacity was first electrodialyzed in the electrodialysis apparatus recently described (17). The electrodialysis was carried out with solutions which were previously dialyzed for 2 days in distilled water. The current at the beginning was around 0.5 milliamperes per square centimeter and subsequently kept below this value. The electrodialysis was continued until the current reached a low value which remained constant. The conductivity of the fluid was then approximately  $3 \times 10^{-6}$  reciprocal ohms.

*8. Hydrogen Ion Activities*—Hydrogen ion activities were determined electrometrically with quinhydrone electrodes at room temperature or in some instances colorimetrically. Each determination was carried out with at least three electrodes and the average was taken from values which as a rule did not differ from each other more than 1 millivolt. The values are given in pH, the standard acetate mixture (0.1 N sodium acetate + 0.1 N acetic acid) was taken to pH 4.605 at 25°.

*9. Determination of Acid-Combining Capacity*—The determination was carried out according to Linderstrøm-Lang (12) in acetone as well as in ethyl alcohol. To 5 cc. of the solution to be titrated 0.5 cc. of a 0.1 per cent alcoholic solution of the indicator was added.<sup>1</sup> Then the amount of acid was added which was determined in a preliminary titration, mixed with 25 cc. of absolute alcohol, and the titration completed. The end-point was compared with a set of standards containing 5 cc. of 0.85 per cent NaCl, 0.5 cc. of indicator, 25 cc. of absolute alcohol (or acetone), and 0.10, 0.20, and 0.30 cc. of 0.1 N HCl. The amount of acid which was present in the standard which matched the end-point in color was subtracted from the value obtained. The average of five determinations was taken. The mean error was  $\pm 0.05$  cc.

*10. Alkali-Combining Capacity*—This was determined by the Willstätter method; titration was carried out with an alcoholic solution of 0.1 N CO<sub>2</sub>-free NaOH in a solution containing 80 per cent alcohol, with thymolphthalein as indicator. 0.5 cc. of a 0.1 per cent alcoholic solution was added to each sample. The standards contained 0.10, 0.15, and 0.20 cc. of alcoholic NaOH. Otherwise the procedure was the same as with the titration with acid. The average of five determinations was taken. The mean error was  $\pm 0.03$  cc.

In spite of the satisfactory consistency of the values obtained by the titration in presence of alcohol, one has to consider a possible systematic error which may arise from the fact that the proteins undergo an irreversible coagulation after the addition of alcohol which may prevent the further binding of acid or base. This error

<sup>1</sup> Naphthyl red was used as the indicator. It was prepared from naphthylamine and diazoaminobenzene as described by Linderstrøm-Lang. The only difference was that the diazoaminobenzene was obtained by the diazotization of aniline with NaNO<sub>2</sub> in acid solution (Gatterman (18)).

is probably not great because the titration is practically completed before the addition of alcohol, the acid or base which is added afterwards serving merely to increase or decrease the hydrogen ion activity to the point where the indicator changes color in the solutions containing 80 per cent of the organic solvent in question.

#### SUMMARY

1. Globulin can be precipitated from normal and immune serum (antipneumococcus) by dilution with distilled water at hydrogen ion activities varying between pH 5.0 to 7.0. The zone of maximum precipitation lies between pH 5.5 and 6.0.

2. By repeated precipitation at pH 5.1 and pH 6.8, respectively, fractions can be obtained from normal serum as well as from immune serum, the pH precipitation curves of which differ from each other, the one being more soluble at neutral, the other at acid reaction.

3. Some physical and chemical properties of these fractions were determined.

4. It has been shown that the fractions combine with each other if their solutions are mixed. The nature of this combination has been discussed.

The writers wish to express their thanks to Dr. Wm. H. Park for his kind interest. They are indebted to Paul Brandwein for valuable assistance in carrying out the experiments.

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## THE NECESSITY OF COPPER AS A SUPPLEMENT TO IRON FOR HEMOGLOBIN FORMATION IN THE PIG\*

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In an earlier paper (1) concerning the value of iron and copper salts in the cure and prevention of anemia in suckling pigs we reported that anemic pigs fed ferric chloride made almost as rapid recovery as those fed both iron and copper. At that time we suggested two possible reasons for these results. First, the iron reserves in the pig at birth may be depleted before the copper store and as soon as iron is supplied hemoglobin synthesis can take place without copper feeding. Second, the pigs were not placed under as restricted conditions as were the smaller animals used in our earlier experimental work (2), and they probably had access to small amounts of copper. We concluded that from a practical point of view the feeding of iron is the most important preventive of anemia in suckling pigs. However, we did not state that copper was unnecessary for hemoglobin formation in the pig. Instead we made the following statement, "it is entirely possible that the importance of copper can be demonstrated by keeping the pigs under very restricted conditions and allowing them to remain anemic for some time to insure a heavy drain on the copper reserves."

Since the publication of this paper several workers have misinterpreted our conclusions and have referred to our work as proof for the fact that not all mammals require copper for hemoglobin formation. For example, Drabkin and Waggoner (3) make the following reference to our work, "It is interesting to note that

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## 364 Cu as Fe Supplement for Hb Formation

Hart and associates . . . have themselves presumably discovered a species (the pig) in which it is difficult to demonstrate the importance of copper. Although all their experiments indicate that iron alone suffices to cure the anemia of young pigs, the Wisconsin workers feel that somehow copper must have been obtained by the animals or else that the pig needs less copper than the rat."

Cunningham (4) also interprets our conclusions to mean that copper is not necessary for hemoglobin formation in the pig. Beard and Myers (5, 6) state that we explain the divergent results obtained with the pig and with the rat by the fact that the copper requirement of the former is not as great as that of the latter. They fail to add, however, that in our experiments with the pigs no attempt was made to keep them under restricted conditions, whereas those with the rats were carefully controlled.

Those who have worked in this field and know the great care that must be taken with rats when copper studies are made certainly should realize the difficulties encountered when similar studies are conducted with pigs. In fact certain workers have found it difficult to control experimental procedures sufficiently to demonstrate the importance of copper in the rat. There are several ways whereby the rat may obtain sufficient copper to obscure the real activity of this element. It is altogether probable that the explanation of the results recently reported by Beard and Myers (5, 6) lies in the milk used. They state ((5) p. 74), "It [milk] was usually obtained from the cows by mechanical milkers, caught in monel metal containers, and immediately taken to the cooler. *It was then poured through about 5 feet of copper pipe<sup>1</sup>* into a large nickel cooler, kept at 7° for 24 hours, bottled, and delivered to the laboratory. Analysis of this milk by the method of Elvehjem and Lindow . . . showed 0.44 mg. of Cu per liter. It would appear, therefore, that the milk used in these experiments did not contain more than the usual amount of Cu."

The amount of copper in normal Holstein milk, such as we have used in all of our experiments, is about one-third of that found by Beard and Myers, namely, 0.15 to 0.2 mg. of copper per liter.

<sup>1</sup> The italics are ours.

Beard and Myers used some milk which did not undergo the above treatment. In reference to the handling of this milk they make the following statement, "In the experiments with Rats 196 to 256, the milk never came in contact with any metal containers. The cows were milked into earthenware jars, the milk bottled, and at once brought to the laboratory. The same results were obtained as with the milk carried through the customary processes." They make no statement as to the copper content of this milk, and it is therefore difficult to compare with the milk produced according to the previous method. We are also unable to find in their published data any records of rats fed the milk and iron only. The records show that this milk was fortified with both iron and zinc, by which the anemia was corrected.

Keil and Nelson (7) had a similar difficulty in their early experiments in demonstrating the need of copper as a supplement to iron for hemoglobin building in the rat. In a later publication (8), in which they duplicated our work completely, they state that the difficulty in their early work in demonstrating the need of copper as a supplement to iron was due to the high content of copper in the milk used; namely, a milk containing 0.35 to 0.44 mg. of copper per liter. In later work they obtained milk from Holstein cows, which, according to their analysis, contained 0.24 mg. of copper per liter. With this milk pure ferric chloride did not correct the anemia, and the anemia was only corrected when copper was added. They state that copper was the *only* element of those tested that had a positive effect on hemoglobin building—a conclusion which is in complete harmony with all of our work.

In our previous work with pigs we took no particular pains to eliminate all copper contamination because we were interested in results of a practical nature and consequently we made no specific conclusions regarding the necessity of copper for hemoglobin formation in this species. In this paper we wish to present results, obtained when iron and copper were supplied to pigs kept under carefully controlled conditions and fed a milk diet, which demonstrate that copper is equally as necessary for hemoglobin synthesis in pigs as it is in experimental animals such as rats, mice, and chickens.

## EXPERIMENTAL

The pigs were farrowed by sows from the general herd. No consideration was given to the diet of the sows before farrowing because we have already shown (1) that the diet of the sow has no effect on the rate at which anemia develops in the young. About a day before parturition the sows were placed in individual pens equipped with wooden floors covered with clean wheat straw. They were then restricted to a diet of whole cow's milk. The young were allowed to suckle the mother until 5 days of age, when they were taken to our animal room and placed in small individual wooden pens. The pens were equipped with sloping floors overlaid with a wooden grating to allow drainage. They were cleaned and washed thoroughly with boiling water every day. The young pigs were given whole cow's milk *ad libitum*. When they were distinctly anemic, iron in the form of purified ferric chloride was added. Copper as copper sulfate was supplied after no further improvement was noted with iron alone. The hemoglobin content of the blood was determined weekly by the Newcomer method with a standardized Bausch and Lomb filter and reported as gm. per 100 cc. of blood.

The hemoglobin values for three of the pigs are given in Chart I. All the pigs developed a severe anemia (3 gm. of Hb per 100 cc. of blood) within 4 weeks after they were placed on the milk diet. When they were continued on this diet for longer periods the hemoglobin decreased to values below 2 gm. per 100 cc. of blood. Some of the pigs were allowed to remain in this anemic condition for some time to deplete the copper reserves as much as possible. These pigs were watched very carefully so that death would not ensue before curative treatment could be established. Pig 601 remained in this very anemic state for 3 weeks before iron feeding was initiated.

The daily addition of 25 mg. of purified  $\text{FeCl}_3$  to the diet of each pig stimulated hemoglobin regeneration to some extent in all cases. The hemoglobin content of the blood increased about 5 gm. in 4 weeks time. However, after 4 weeks of iron feeding no further improvement was noted and in most cases a decided drop in the hemoglobin values followed. The values for Pig 360 dropped to about 3 gm. of hemoglobin per 100 cc. of blood although 25 mg. of iron were supplied daily. Pig 601 was given 10 mg. of

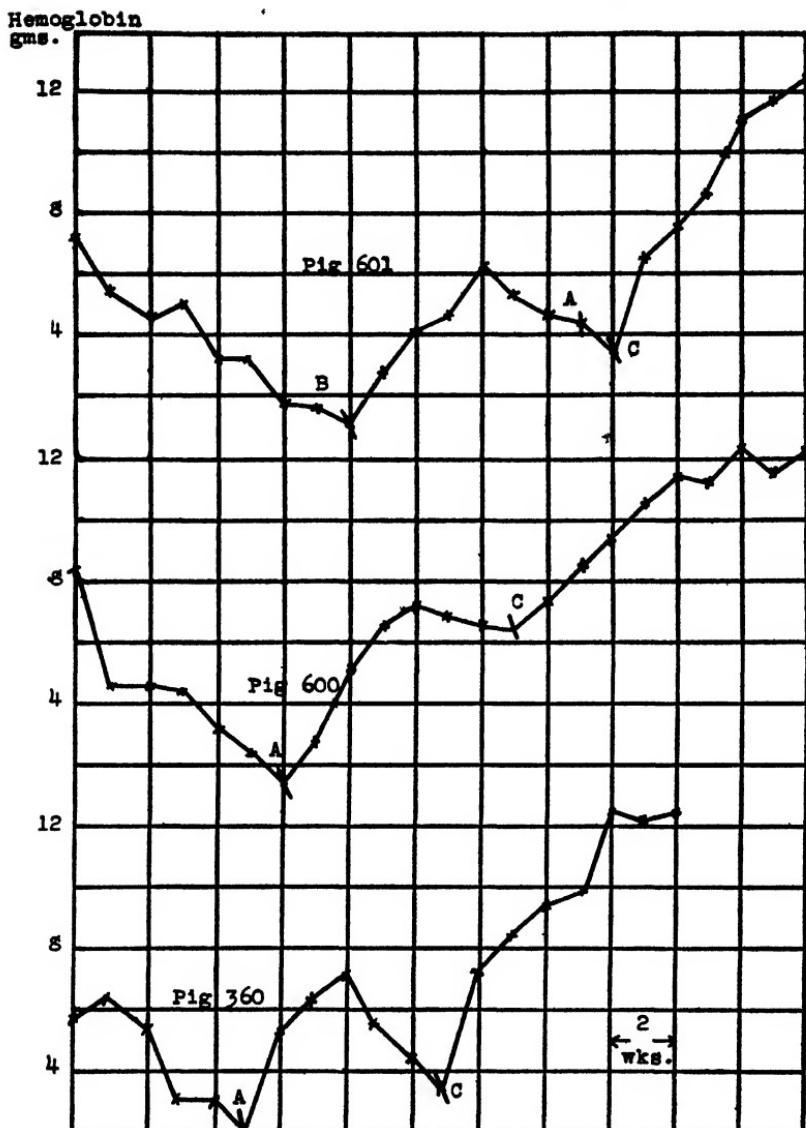


CHART I. Results secured when iron and copper additions were made to the diets of anemic pigs maintained on whole cow's milk. *A* represents the initiation of a supplement of 25 mg. of iron alone; *B*, a supplement of 10 mg. of iron alone; and *C*, a supplement of 25 mg. of iron plus 5 mg. of copper. The additions were made 7 days per week. The solid lines represent hemoglobin in gm. per 100 cc. of blood.

iron at first but a similar small improvement was noted with the typical decrease after 4 weeks. This decrease was not retarded by increasing the daily iron intake to 25 mg.

When 5 mg. of copper were supplied in addition to the iron, rapid and pronounced improvement was noted in all pigs. In the case of Pigs 360 and 601, the hemoglobin values increased from 3.6 to 12 gm. per 100 cc. of blood in a period of 5 to 6 weeks. A decided improvement in the activity of the pigs was also noted during this period. The pigs became so large at this stage that it was impossible to continue them on iron and copper for longer periods, but there was no indication that the hemoglobin values would not continue at this level. These results demonstrate that copper is necessary for hemoglobin formation in the pig as well as in the other animals that have been studied.

#### DISCUSSION

The papers by Beard and Myers (5, 6) which appeared in a recent number of this *Journal* in which they concluded that inorganic iron *per se* prevents and cures nutritional anemia in young rats may raise a question in the minds of workers primarily interested in other fields as to the reliability of our conclusions. Suffice it to say that our demonstration of the importance of copper has now been verified in seven laboratories by different workers; *viz.*, McHargue, Healy, and Hill (9), Krauss (10), Titus, Cave, and Hughes (11), Lewis, Weichselbaum, and McGhee (12), Underhill, Orten, and Lewis (13), Cunningham (4), and Keil and Nelson (8).

Myers and Beard refer to the fact that their results are in agreement with those of Keil and Nelson (7), Drabkin and Waggoner (3), and Mitchell and Schmidt (14). In this connection it is interesting to note that Keil and Nelson (8) in a recent paper explain that their earlier results were due to the high copper content of the milk used and state that their present data obtained under more carefully controlled conditions convincingly demonstrate that copper possesses the unique power of hemoglobin formation demonstrated by Waddell, Steenbock, and Hart (15). Although Drabkin and coworkers do not agree that copper is unique in this connection they do find that pure iron added to a milk diet does

not stimulate hemoglobin formation. It is true that Mitchell and Miller (16) in a recent paper, report some improvement in the hemoglobin of anemic rats when iron is fed alone, but they do not conclude that copper is without a rôle in the synthesis of the blood pigment. Rather, they make the following statement, "In the present instance when the iron salt was of such purity as to preclude possibility of significant copper contamination it seems most logical to attribute the slow response observed to the traces of copper available from the milk supply or stored in the animal body." Since Beard and Myers observed hemoglobin regeneration in the rats kept under their régime when iron was supplied in the form of purified iron solutions prepared in this laboratory and by Dr. Krauss of the Ohio Agricultural Experiment Station, it seems justifiable to apply one or both of Mitchell and Miller's explanations to the results obtained by Beard and Myers.

We have suggested in our earlier papers (17) that the recoveries obtained by Mitchell and Schmidt (14) and Beard and Myers (18) might be due to impurities such as copper in the iron salts used. Naturally we would make this suggestion since this is the only source of copper contamination that we have encountered in our work with rats. Other laboratories may find that copper contamination is more likely to occur from other sources.

Myers and Beard also make reference to our work with chicks (19) and state that we were able to cure anemia in chicks with pure  $\text{FeCl}_3$ . It is true that we were able to do this when certain basal rations were used, but we reported in the same paper that if the basal ration were low enough in copper the anemia could not be cured with iron alone. The situation there was obviously the same as we have now demonstrated it to be with the pig.

From the experience in this laboratory and from all the results reported in the literature we believe that we are justified in concluding that when hemoglobin regeneration takes place with the addition of iron alone the animal either contains, or is receiving sufficient copper for this purpose from some other source.

So far we have demonstrated that copper is necessary for hemoglobin formation in rats, chickens, mice (unpublished data), and pigs. What the situation is for other animals remains to be demonstrated.

## SUMMARY

When pure iron was added to the whole milk diet of anemic pigs kept under restricted conditions, there was a small temporary improvement in the hemoglobin content of the blood, but a rapid and complete recovery was obtained only after copper was supplied in addition to the iron.

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## EFFECT OF HEAT AT VARYING CONCENTRATIONS OF HYDROGEN ION ON VITAMIN G ( $B_2$ ) IN PROTEIN- FREE MILK\*

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Vitamin G ( $B_2$ ) has been designated as the more heat-stable member of the group of B vitamins, but the stability to heat appears to be dependent, among other things, on the length of time of heating and the hydrogen ion concentration of the medium—the more acid the solution, in general, the greater is the stability, and at the same reaction the amount of destruction seems to be dependent upon the length of time of heating.

Guerrant and Salmon (1) found that dry heating or autoclaving a yeast extract at pH 2.7 or pH 6 caused almost 20 per cent destruction of vitamin G, while at pH 10.1 there was fully 75 per cent destruction. Williams, Waterman, and Gurin (2) found very little vitamin G left after autoclaving brewers' yeast for 6 hours at pH 12 to 14, while some loss had occurred at pH 8. Reader (3) reported no destruction of the vitamin in marmite when heated for 1 hour at pH 9 at 120°. Chick and Roscoe (4) reported the following results. Heating yeast extract or marmite 2 hours at pH 5 (90–100°) caused no loss, 4 hours heating at 123° at pH 3 to 5 gave 50 per cent loss, 2 hours at 90–100° at pH 8.3 caused 50 per cent loss, while 4 to 5 hours at 122° at pH 10 to 8.3 caused 75 to 100 per cent destruction.

The present investigation was undertaken to determine whether

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similar results would be obtained when a protein-free milk known to be relatively rich in the vitamin was used. This material was chosen because it has been well standardized, is easily prepared, and is more nearly related to natural foods than the yeast extracts commonly used in this work.

Methods of conducting these experiments and evaluating results vary to some extent in different laboratories. Different materials are used as the source of the antineutritive vitamin, and various diets have been suggested in the effort to obtain one adequate in all known respects but deficient in this one factor. The length of the experimental period and the best rate of growth to be adopted have been discussed.

The preparation used as a source of vitamin B ( $B_1$ ) may carry some vitamin G ( $B_2$ ). In this case it is possible to consider the gain of experimental animals above that made by litter mate, negative controls (animals receiving the basal diet only) as the correct measure of the vitamin in the test material.

An experimental period of 4 or 5 weeks instead of 8 has been suggested by Alleman (5) since the animals ceased to grow at a constant rate after about the 5th week in the experiments which she described. Akyroyd and Roscoe (6) have suggested discarding the 1st week's gain and using that of the 2nd to the 5th week, since animals often show a variable reaction at first.

Two "units" have been proposed, one by Roscoe (7) and Chick and Roscoe (8) and the other by Bourquin and Sherman (9). The former suggest as the criterion that amount of test material which will induce a gain of 50 to 60 gm. in 5 weeks. The latter authors found that a lower rate of gain (about 3 gm. per week) during a period of 4 to 8 weeks tends to lessen the danger that "the quantitative accuracy of the experiment will be injured by exhaustion of the bodily store of any growth-essential not yet sufficiently known to be provided in the basal diet."

Roscoe (7) and Chick, Copping, and Roscoe (10) have recently advocated cure of dermatitis in rats as a means of assay.

In order to compare these methods the results obtained in this present work were evaluated, as to (1) gain in weight of the experimental animals in 8 weeks above that made by litter mate, negative control rats; (2) gain made by the experimental animals during the first 4 weeks of the experiment; (3) gain made by the

experimental animals during the 2nd to the 5th week of the experiment; and (4) potency of the materials in curing dermatitis in rats.

#### EXPERIMENTAL

The plan of the work was to determine the vitamin G value of (a) skim milk powder, (b) protein-free milk, prepared from this skim milk powder, at its natural acidity (about pH 4.3) as well as after it had been brought to pH 7 and pH 10, and (c) these solutions after 1 or 4 hours of heating in a water bath at about  $97 \pm 1^\circ$ . By this means the percentage destruction due to heat, change in concentration of hydrogen ion, or a combination of these two factors could be determined.

The protein-free milk was prepared as follows: Skim milk powder was mixed with distilled water and such a volume of 1 per cent HCl added as to precipitate the casein as completely as possible. The mixture was boiled for 5 minutes, cooled, and filtered. This solution had a reaction of about pH 4.3. One aliquot was maintained at this acidity, one aliquot was brought to pH 7, and one to pH 10 by the use of 0.2 M NaOH. One portion of each of these solutions was heated 1 hour and one 4 hours in a water bath. The material was prepared fresh weekly.

All E. M. F. determinations were made electrometrically, with carefully platinized hydrogen electrodes and a saturated calomel cell.

The technique described by Bourquin and Sherman (9) was used throughout, with this exception. The casein was extracted with cold dilute acetic acid for 1 week, with the acid solution changed daily. It was dried and ground and then extracted for 1 hour in boiling 95 per cent alcohol.

All portions were fed daily, except Sunday, in amounts corresponding to a weight of skim milk powder, the vitamin content of which had been determined.

After the depletion period litters of rats were so distributed that litter mates received supplements from different materials or graded portions of the same materials. The sex and weight of the rats receiving various supplements were as uniformly distributed as possible.

*Results*

In Fig. 1 are shown curves representing the growth of experimental animals above that made by litter mate, negative controls,

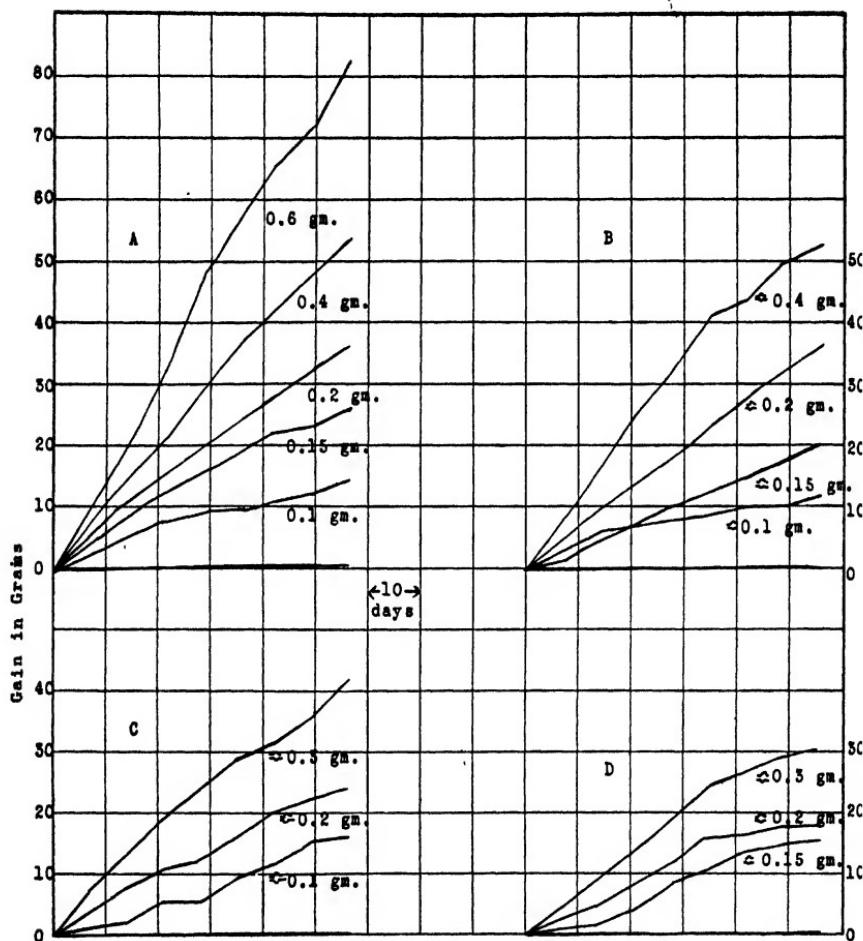


FIG. 1. Curves representing average gain of rats above that made by litter mate negative controls when they received the vitamin G- ( $B_2$ ) deficient diet plus (Group A) graded portions of skim milk powder, (Group B) graded portions of protein-free milk at pH 4.3, (Group C) graded portions of this solution heated 1 hour, and (Group D) graded portions of this solution heated 4 hours. Each curve represents the average of results obtained from six or more rats.

when graded portions of skim milk powder, protein-free milk at pH 4.3, and this solution heated 1 or 4 hours were fed to rats which had been depleted of their store of the vitamin. There seemed to be no demonstrable loss of vitamin potency in the preparation of the protein-free milk and only about 10 per cent on heating 1 hour at this acidity. On heating 4 hours two things became apparent. There was a loss in vitamin potency, and the flattening of the curves after about the 5th week seemed to indicate a lack of some factor which would influence growth.

Fig. 2 shows the same results when the actual gain of the animals is charted. The animals receiving skim milk powder continued to grow at a constant rate throughout the 8 weeks. Those receiving unheated protein-free milk showed a decline in growth rate after about the 5th week, and this was more pronounced in animals receiving heated solutions. Apparently this second factor is very heat-labile even in an acid medium. The flattening of the growth curves is not always evident when the growth above that made by negative control animals is plotted. Most of the negative control rats lost weight during the last 3 to 4 weeks of the 8 weeks period, so that the relative gain made by experimental animals remained more constant.

Although the absolute and relative growth curves do not correspond exactly, the same conclusions can be drawn; *i.e.*, that there was no demonstrable loss of the vitamin in preparing the solution, about 10 per cent was destroyed in heating it 1 hour, and 4 hours of heating led to about 30 per cent loss. This is evident when the gain either during the first 4 weeks or from the 2nd to the 5th week is considered.

A curve showing the average growth of forty-nine negative controls is shown in Fig. 2, and for comparison are given the curves obtained by the author (11) in previous work in another laboratory and that reported by Bourquin and Sherman (9). In spite of a large individual variation among the animals, the final results were similar and the growth curves closely parallel. It seems evident that fairly reproducible results can be obtained by this method.

Since the average growth curve of all the negative control rats showed a loss during the experimental period it would seem that the gain of experimental animals above that made by the negative

controls should be greater than the actual gain. That this is not always the case is seen by a comparison of the growth curves. It is due to the fact that many negative control rats continued to gain in weight during the experimental period, a gain of 20 gm. not being exceptional. This would naturally affect the average result to some extent. An example of this is seen in the curve representing the growth of animals receiving 0.4 gm. of skim milk powder daily. The average of the actual gains was 57 gm., while the gain above that of the litter mate negative control rats averaged about 53 gm.

Fig. 3 shows the results obtained with the solution brought to pH 7 and a comparison made according to the three criteria, the gain made above that of litter mate negative controls, actual gain in 4 weeks, or gain during the 2nd to the 5th week. Here again the actual gain was in some groups greater than the relative, but a consideration of the results makes it evident that the same conclusions can be drawn from either set of curves. It seems evident that there was about 10 per cent loss of the vitamin when in a solution at pH 7 unheated. Heating for 1 hour caused a loss of about 30 per cent in this case, with a greater loss on heating 4 hours. A comparison of the vitamin content of the two solutions heated 4 hours showed a loss of about 30 per cent at pH 4.3 and fully 50 per cent at pH 7.

Fig. 4 shows similar results with the solutions at pH 10. In this case a complication arose which made it impossible to obtain a direct comparison of the three solutions. When the solutions were heated 1 hour the concentration of hydroxyl ion dropped from pH 10 to about pH 8.0. After 4 hours there was a further drop to about pH 7.0. The unheated solution remained at pH 10.

FIG. 2. (1) Average growth curves of rats receiving the vitamin G- ( $B_2$ ) deficient diet alone, or this diet plus (Group A) graded portions of skim milk powder, (Group B) graded portions of protein-free milk at pH 4.3. (2) Average growth curves of rats during the first 4 weeks of the experimental period when they received (Group A) graded portions of skim milk powder, (Group B) graded portions of protein-free milk at pH 4.3, (Group C) graded portions of protein-free milk at pH 4.3 heated 1 hour, and (Group D) graded portions of protein-free milk at pH 4.3 heated 4 hours. (3) Average growth curves of rats during the 2nd to the 4th week of the experimental period when they received these supplements. Each curve represents the average of results from six or more rats.

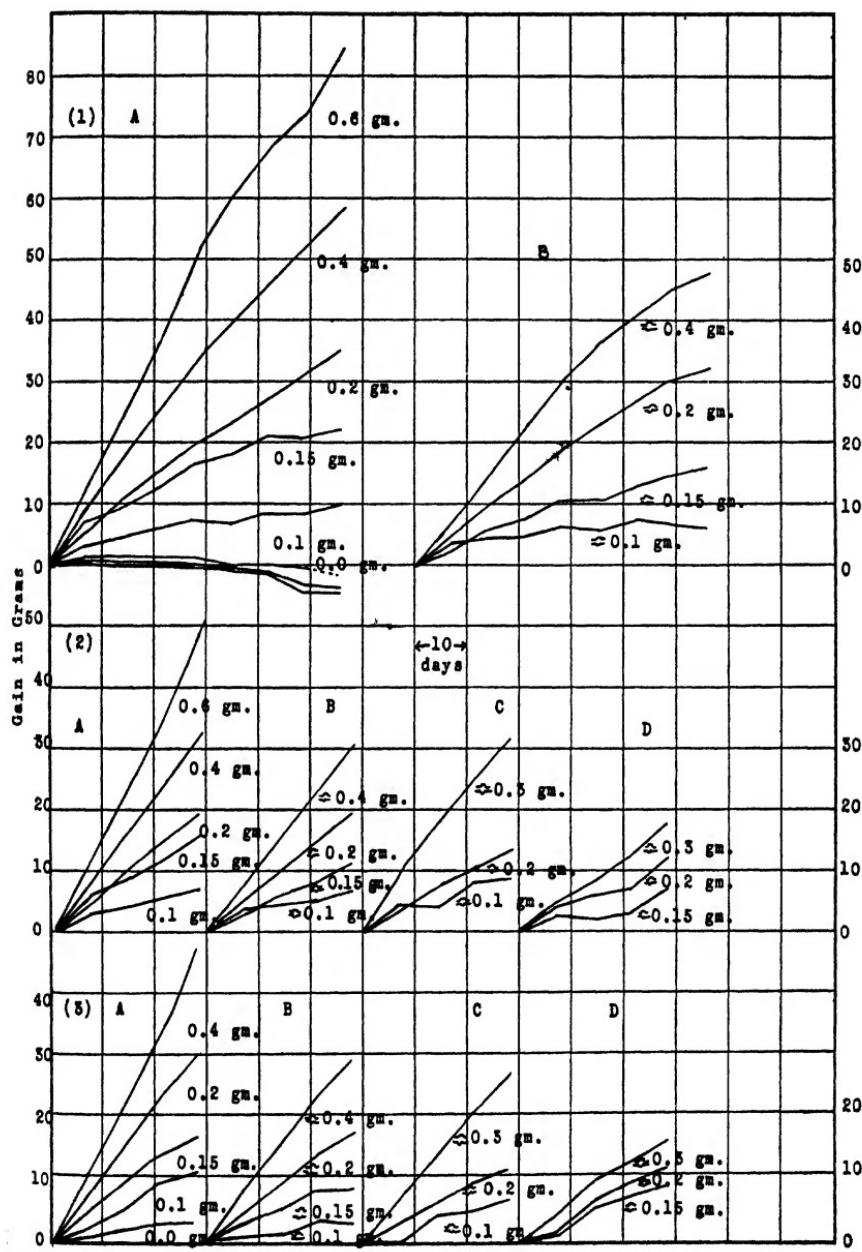


Fig. 2

## Effect of Heat on Vitamin G

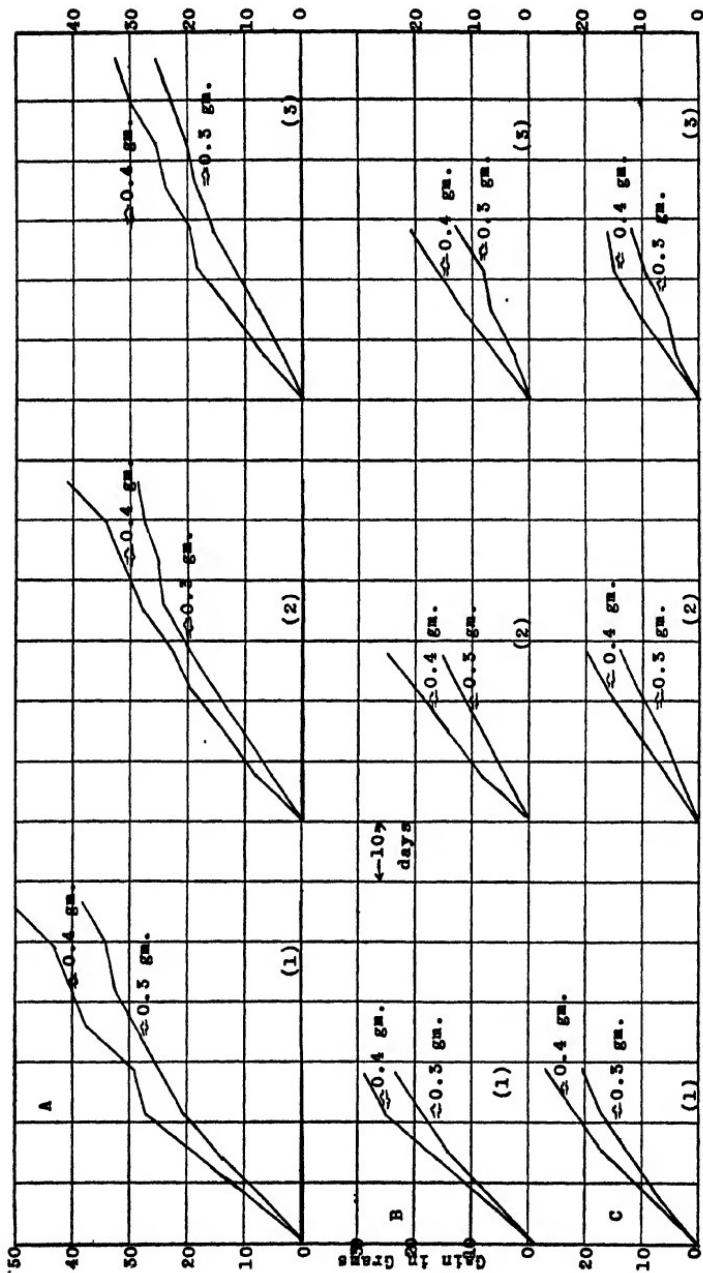


Fig. 3. Group A, curves showing average gain of rats above that made by litter mate negative controls when they received the vitamin G- $(B_2)$  deficient diet plus graded portions of protein-free milk at pH 7 (1) unheated, (2) heated 1 hour, and (3) heated 4 hours. Group B, curves showing average gains made by these rats during the first 4 weeks of the period. Group C, curves showing average gains during the 2nd to the 5th week of the period. Each curve represents the average of results from six or more rats.

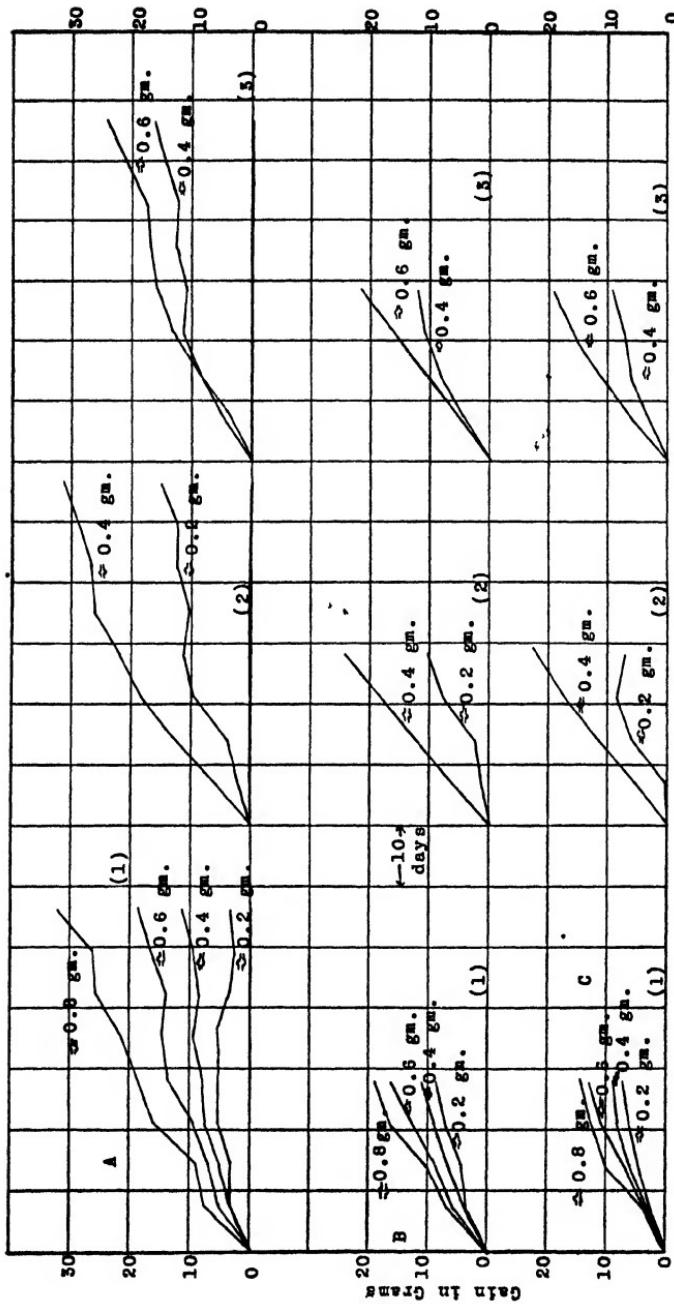


Fig. 4. Group A, curves showing average gain of rats above that made by litter mate negative controls when they received the vitamin G-<sub>(B)</sub> deficient diet plus graded portions of protein-free milk at pH 10 (1) unheated, (2) heated 1 hour, and (3) heated 4 hours. Group B, curves showing average gains made by these rats during the first 4 weeks of the period. Group C, curves showing average gains during the 2nd to the 5th week of the period. Each curve represents the average of results from six or more rats.

throughout the week. As a result there was a greater loss of vitamin potency in the unheated solution than in the solution heated 1 hour, and the material heated 4 hours showed about the same activity as the unheated. (The solutions were held about 18 hours before heating, thus allowing for some destruction due to the higher concentration of alkali.) These results were similar to those found by Sherman and Burton (12) for tomato juice and Chick and Roscoe (8) for yeast extract. In the former case the concentration of hydroxyl ion dropped from pH 10.9 to pH 8.5 after heating 4 hours, while in yeast extract there was a drop from pH 10 to pH 8.2 after 4 hours autoclaving.

Attempts have been made to buffer the solution at pH 10 but the usual buffer systems have not been found to be effective. However, this is being investigated further.

The results with the three criteria show about 70 per cent destruction of vitamin G at pH 10 in the cold. There was about 40 per cent loss after heating 1 hour at pH 10 to 8 as compared with 30 per cent at pH 7 and 10 per cent at pH 4, while after 4 hours of heating at pH 10 to 7 almost three-fourths of the vitamin was lost.

All of the animals receiving these more alkaline solutions showed a marked flattening of the growth curves after the 5th week. For example the animals receiving the solutions heated 1 hour at pH 10 to 8 in amounts corresponding to 0.4 gm. of skim milk powder reached an average weight of 29.2 gm. at the end of the 5th week and only 33.9 gm. in 8 weeks, indicating a loss of this second factor in the alkaline solution.

Since the rate of growth attained in the experiments described is presumably a measure of the vitamin potency of the material tested, and furthermore, since all liquid supplements were fed in such amounts as to correspond to a weight of skim milk powder, the vitamin content of which was known, it is possible to determine the degree of destruction of the vitamin by comparing the amounts of the various supplements which induce the same gain in the animals used. In Table I are given these results. If the portions of skim milk powder required to induce certain gains are given a value of 1, the untreated protein-free milk at pH 4.3 has the same value, while it is necessary to feed slightly more of the material at pH 7 and 3 to 4 times as much at pH 10. The potency and percentage loss are given.

A comparison may be drawn here of the three criteria used. It will be seen that there is close agreement and that the same conclusions could be drawn from either set of data. If the basal diet appears to carry an appreciable amount of the vitamin, the gain over the litter mate, negative control would be a more accurate measure of the potency of the material tested. If on the other hand the actual gain is used as the criterion, the experimental period should end when the rate of growth slackens. In these

TABLE I  
*Showing Relative Amounts of Various Protein-Free Milk Preparations Which Induced Same Rate of Gain As Skim Milk Powder, Taken As Unity, If Gain over Negative Controls, Gain during First 4 Weeks, or Gain during 2nd to 5th Week Is Considered*

Supplement	Gain over negative controls	Gain during 1st 4 wks.	Gain during 2nd to 5th wk.	Average	Potency per cent	Destruction per cent
Skim milk powder.....	1	1	1			
Protein-free milk						
pH 4.3, unheated.....	1	1	1	1	Equal	None
" 4.3, 1 hr.....	1.1	1.1	1.1	1.1	90	10
" 4.3, 4 hrs.....	1.5	1.4	1.4	1.4	70	30
" 7, unheated.....	1.2	1	1.3	1.2	85	15
" 7, 1 hr.....	1.3	1.4	1.8	1.5	67	30
" 7, 4 hrs.....	2.3	2.0	2.1	2.1	50	50
" 10, unheated.....	3.0	4.0	4.0	3.7	25-30	70-75
" 10, 1 hr.....	2.3	1.4	1.5	1.7	60	40
" 10. 4 hrs.....	3.5	3	3.2	3.2	30-45	65-70

experiments the growth appeared to be more consistent during the 2nd to the 5th week than during the first 4 weeks.

If the cure of dermatitis lesions was used as a criterion there was little correlation with the results just reported, perhaps, because in the cases about to be described, the animals were too far weakened by the vitamin G deficiency to permit of their making good recoveries.

When the animals received skim milk powder or the solutions of protein-free milk in amounts inducing about 3 gm. a week gain or more the fur was good and no sores were seen, although their litter

mates receiving lesser amounts or no supplements in almost every case showed dermatitis.

For the curative tests the negative control rats were used after they had received the vitamin-deficient diet about 8 to 10 weeks. If the condition was mild it was improved or cured and growth was resumed in every case when the protein-free milk at pH 7 was fed. With the solution at pH 4.3 the animals grew but in some cases the dermatitis became steadily worse. Three animals receiving this material gained 10 to 12 gm. a week for 8 weeks but at the end of this period one rat had lost three toes and one ear, while the others showed bald patches which had developed into raw areas spreading around the axilla and down the foreleg. The animals receiving solutions at pH 10 did not grow nor did the dermatitis improve.

Some animals were continued on the basal diet alone until their conditions became very severe. The feet were edematous and large ulcers appeared on the pads. In many cases one or more digits were lost and in some one or both ears. There was no fur on forelegs or chest and the skin became raw. These animals were given either skim milk powder or one of the solutions, but in no case was a cure effected, although there was an increase in weight.

In order to use this method it would seem necessary to determine the stage to which the condition should be allowed to develop. Since animals receiving the same diet and coming from mothers which had been given the same laboratory stock diet showed large variations in the time required to develop the condition, it was not possible to use age or weight as a guide. The results in these experiments could not be considered quantitative. It may be possible that the test materials which the English workers used contained a factor lacking in skim milk powder, or that the animals used by them were in early stages of the condition. In no case were our animals completely cured in 3 weeks even when they received portions of the protein-free milk which had induced good gain and absence of any dermatitis symptoms in experimental animals.

#### DISCUSSION

The experiments just described indicate that vitamin G in protein-free milk is sensitive to heat and to alkali—as much as 70 per cent of the original potency being lost by heating 4 hours at pH 10

to 7 or by holding in the cold at pH 10. The rate of destruction at any reaction increases with time of heating, while heating the same length of time will cause greater destruction the more alkaline the solution. Whether or not this destruction is due to oxidation is being investigated. Sherman and Sandels (13), Bisbey (14), Halliday (11), and others found vitamin G rather more easily oxidized than vitamin B ( $B_1$ ).

There seems to be evidence that skim milk powder possesses some factor or factors which induce a steady rate of growth, and which are lost in manipulation processes. The loss was greater in more alkaline solutions and after a longer period of heating in acid solutions. This loss resulted in a flattening of the growth curves after about the 5th week. The possible identity of this factor is being studied. As a result it seems best to draw conclusions from the weight attained at 4 or 5 weeks.

As previously stated, the diet used in these experiments included casein extracted in dilute acetic acid and then in boiling 95 per cent alcohol. Chick and Roscoe (8) and more recently Goldberger and associates (15) have stated that this vitamin is not readily extracted in acetic acid. This may have accounted for the irregularity found among the negative control rats. A small group of animals which are receiving a diet including casein extracted in cold 60 per cent alcohol, according to the method of Sherman and Spohn (16), appears to be more consistent. Because of the irregularity of growth in the experiments just described the relative rather than the absolute gain may give a truer indication of the potency of the materials.

Conclusions based on cure of dermatitis did not agree with those drawn from the growth rate. It was found possible to induce growth in animals suffering from severe dermatitis without curing the condition by feeding unheated protein-free milk at pH 4.3, while the solution at pH 7 (unheated) appeared to be more potent. The solutions at pH 10 had no potency.

The heat stability of this vitamin thus again is shown to vary with time of heating and hydrogen ion concentration of the medium. However, Guha (17) has shown recently that the stability, like that of vitamin B ( $B_1$ ), varies in different media. He showed that autoclaving an aqueous extract of fresh ox liver at pH 9 (124–125°) caused about 75 per cent inactivation, a brewers' yeast

extract was about 90 per cent inactivated, while there was no apparent loss of vitamin potency when an extract of commercial liver concentrate was autoclaved under these conditions.

#### SUMMARY

1. Experiments are described in which the stability of vitamin G to heat and alkalinity was studied. The technique of Sherman and Bourquin was followed. Determinations were made of the vitamin content of skim milk powder, protein-free milk at pH 4.3 and after it had been brought to pH 7 and to pH 10, and these solutions heated 1 or 4 hours in a water bath at  $97^{\circ} \pm 1^{\circ}$ .
2. The vitamin appears to be completely extracted from the milk powder into the protein-free milk at pH 4.3.
3. Heating 1 hour at pH 4.3, pH 7, or pH 10 to 8 caused 10 per cent loss in the first case, 30 per cent loss in the second, and about 40 per cent at pH 10 to 8.
4. Heating 4 hours caused 30 per cent loss at pH 4.3, 50 per cent at pH 7, and about 75 per cent at pH 10 to 7.
5. Holding the solution 1 week in the cold at pH 4.3 and pH 7 caused practically no loss, but at pH 10 fully 75 per cent of the vitamin was destroyed.
6. Evidence is presented that some factor for growth present in the skim milk powder is destroyed to a small extent in preparing the protein-free milk. The factor appears to be very heat-labile and sensitive to alkali.
7. Cure of dermatitis could be used only as a qualitative test and the results did not in general agree with those obtained by the growth method.
8. A discussion of methods of assay of the vitamin is included.

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# PURIFICATION OF BENZIDINE, AND AN IMPROVED REAGENT, FOR ESTIMATING HEMOGLOBIN IN BLOOD

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In the quantitative determination of hemoglobin by the method of Bing and Baker<sup>1</sup> it is essential to use a very good grade of benzidine. Recently, we have encountered several samples, including expensive products labeled "suitable for detecting blood," that were unsatisfactory. Some of these were not much improved by the method of purification previously described. One manufacturer specializing in fine quality chemicals has written us that his concern had no preparation in stock which gave a negative blank when our directions for testing the reagent were followed. After a considerable number of trials we have succeeded in developing a simple process for obtaining a suitable reagent from all samples of benzidine that we have tried. Needless to say, such preparations should also prove satisfactory for the qualitative detection of blood.

The following method of purification depends upon the fact that the interfering materials in most commercial specimens of benzidine are quite soluble in 50 per cent alcohol. Dissolve 20 gm. of benzidine base in 200 cc. of ordinary ethyl alcohol, with the aid of gentle heating. Filter off the insoluble material. Add 1 gm. of blood charcoal (extracted with HCl and reactivated) to the filtrate, stir, and maintain at a temperature of  $50^{\circ} \pm 10^{\circ}$  for 15 minutes. This can best be done on an electric hot plate. Filter while warm and wash the charcoal with 10 cc. of warm alcohol. Repeat the treatment with charcoal until the filtrate is almost colorless. With benzidine of fair purity two to three additions of charcoal usually

<sup>1</sup> Bing, F. C., and Baker, R. W., *J. Biol. Chem.*, **92**, 589 (1931).

will suffice, but with highly colored products ten to twelve treatments may be necessary. To the filtrate add 135 cc. of distilled water, or sufficient water to make the alcohol 50 to 60 per cent by volume, and warm if necessary to obtain a clear solution. Allow the solution to stand in the refrigerator for 24 to 48 hours to crystallize the benzidine. Filter on a Buchner funnel with suction, wash with cold 50 per cent alcohol, and continue the suction until the crystals are dry. Preserve the benzidine in a bottle protected from light. From 20 gm. of a highly colored technical product 12 gm. of purified material were obtained.

All benzidine so prepared by us has given a negative blank when tested according to the following directions. Dissolve, with the aid of heat, 1 gm. of purified benzidine in 20 cc. of glacial acetic acid. Add 30 cc. of distilled water and 50 cc. of ordinary 95 per cent alcohol. This constitutes the reagent that we now use in our hemoglobin determinations. The solution may have a trace of yellow color but decolorization with charcoal at this stage is neither necessary nor desirable. Place 2 cc. of the reagent in a test-tube, add 1 cc. of distilled water and 1 cc. of 0.6 per cent  $H_2O_2$ , and mix. No color, or only a very slight yellow color, should develop in 2 hours. If this mixture is then diluted to 25 cc. with 20 per cent acetic acid and viewed through a thickness of about 1 cm. it should be practically indistinguishable from distilled water.

The addition of alcohol to the benzidine-acetic acid reagent has a distinct purpose. Some non-alcoholic solutions of benzidine, even though producing negative blanks, do not show a straight line proportionality between color development and hemoglobin concentration. The addition of an equal volume of 95 per cent alcohol to the benzidine-acetic acid mixture produces correct results. Incidentally, the new reagent gives with blood exactly the same amount of color as the old, even though the concentration of benzidine is halved.

For hemoglobin determinations the technique of Bing and Baker is followed, except that 2 hours are allowed for full color development. Repeated experiments have shown that 75 per cent of the final color value is obtained in 20 minutes, 96.5 per cent in 60 minutes, 99.7 per cent in 100 minutes, and 100 per cent in 120 minutes. The reaction mixture may be allowed to stand for several hours before dilution to 25 cc., if desired.

## THE MECHANISM OF SULFUR LABILITY IN CYSTEINE AND ITS DERIVATIVES

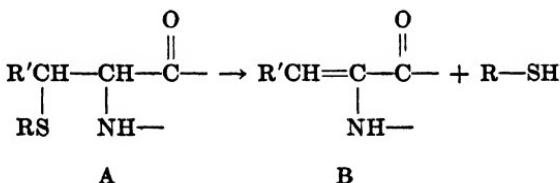
### II. THE ADDITION OF MERCAPTAN TO BENZOYLAMINOCINNAMIC ACID DERIVATIVES

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(Received for publication, December 28, 1931)

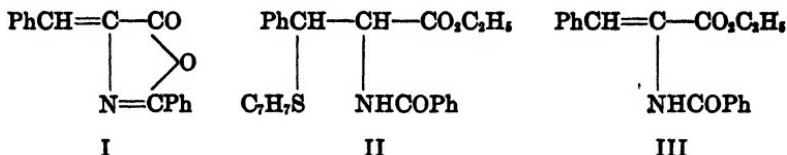
In the case of a number of derivatives containing the grouping (A), in which R and R' may be either hydrogen or some radical, it



has been demonstrated that, usually under the influence of alkalies, R-SH may be eliminated to leave a derivative (B) of aminoacrylic acid. Such a reaction product may be quite stable (particularly in those cases in which formulas (A) and (B) represent cyclic structures (1-3)) or, as in the simplest case of cysteine (4, 5), may at once undergo further decomposition. Certain reactions of the type indicated, involving substances in which suitable groups are attached to the NH and CO groups as represented in formula (A), take place with extreme ease (6).

The writer has discussed elsewhere (6) in some detail the decomposition of cysteine by alkalies. In the light of the mechanism there considered, there was reason to believe that such reactions would, under proper conditions, be reversible, and the present paper describes a demonstration that this is indeed the case.

The substance chosen was 2-phenyl-4-benzal-5-oxazolone (I).<sup>1</sup>



When this is treated in benzene with *p*-tolylmercaptan and a small amount of sodium ethylate in ethyl alcohol, reaction readily takes place with addition of the mercaptan and also of alcohol, forming ethyl  $\alpha$ -benzoylamino- $\beta$ -*p*-tolylmercapto- $\beta$ -phenylpropionate (II). This substance might also be called N-benzoyl-S-*p*-tolyl- $\beta$ -phenylcysteine ethyl ester. With the use of other alcoholates, other esters are formed.

Under the conditions described above, except that the mercaptan was omitted,  $\alpha$ -benzoylaminocinnamic ester was obtained in good yield.<sup>2</sup> This, in turn, could also readily be made to add tolylmercaptan to form the ester (II), so that evidently the cyclic character of the azlactone is in no way essential for the mercaptan addition.

Attempts to add hydrogen sulfide instead of the mercaptan, and thus obtain a product more closely related to cysteine, have not as yet been completely successful. Evidence that somewhat analogous additions of hydrogen sulfide can occur has, however, been obtained, and will be presented in a later paper.

To the writer, it seems evident that a synthesis of cysteine itself should be possible, based on the addition of hydrogen sulfide to some suitable derivative of aminoacrylic acid,  $\text{H}_2\text{C}=\text{C}(\text{NH}_2)\text{CO}_2\text{H}$ , and work in this direction is being undertaken. Aminoacrylic acid itself is much too unstable for the purpose, and it will obviously be necessary to modify suitably both the carboxyl and the amino groups, the latter in the interest of increased stability, and the former for increase in the additive capacity of the double bond (6). It is not difficult to imagine an attractive biochemical

<sup>1</sup> 2-Phenyl-4-benzal-5-oxazolone (I) is hereafter called, for brevity, the azlactone.

<sup>2</sup> This method is at least as convenient as that of Erlenmeyer (7) which involves acid alcoholysis.

synthesis along such lines, but a detailed discussion of this possibility is postponed until further experimental results can be obtained.

#### EXPERIMENTAL

*Ethyl α-Benzoylamino-β-p-Tolylmercapto-β-Phenylpropionate (II)*—5 gm. of 2-phenyl-4-benzal-5-oxazolone (7) and 2.6 gm. of *p*-tolylmercaptan in 40 cc. of benzene were treated with 1 cc. of 2 N sodium ethylate solution. After an hour at room temperature (the solution may be warmed gently if the azlactone has not dissolved completely, but this should not be necessary) the reaction was stopped by the addition of a slight excess of acetic acid, and the benzene removed by evaporation. The white solid, after a few recrystallizations from alcohol, melted at 136°.

$C_{26}H_{22}O_4NS$ . Calculated. S, 7.64  
Found. " 7.70, 7.80 (Parr bomb)

The same product was obtained when ethyl- $\alpha$ -benzoylamino-cinnamate (III) was condensed with *p*-tolylmercaptan substantially as described above (the mixture in this case was warmed gently for 1 hour, but it was not shown that this was necessary).

*Preparation of Methyl Ester*—The condensation of the azlactone was carried out as described above, except that a solution of sodium methylate in methyl alcohol was used as the condensing agent. The crude product was obtained in good yield, but was not readily purified by crystallization from methyl alcohol. After several crystallizations from 75 per cent acetic acid it melted somewhat sluggishly at 130–132°, and did not give a completely clear melt. A mixture with the ethyl ester melted at 126°.

$C_{24}H_{22}O_4NS$ . Calculated. S, 7.90  
Found. " 8.11, 8.21 (Parr bomb)

*Butyl Ester and Its Oxidation*—When sodium butylate in *n*-butyl alcohol was used as the condensing agent, the product was an oil, which in the ice box became almost a glass. For characterization, it was dissolved in chloroform and oxidized by shaking with an acidified 2 per cent solution of potassium permanganate. The resulting product crystallized well from alcohol, and melted at 136°. It was presumed to be the sulfone of the butyl ester.

C<sub>27</sub>H<sub>28</sub>O<sub>6</sub>NS. Calculated. S, 6.68  
Found. " 6.95, 7.08 (Parr bomb)

*Ethyl α-Benzoylaminocinnamate*—When the azlactone (1.0 gm.) was dissolved in 15 cc. of benzene, treated with 0.3 cc. of 2 N sodium ethylate solution in absolute alcohol, and kept for 1 hour at room temperature, ethyl α-benzoylaminocinnamate was easily isolated in good yield from the acidified solution. This method is quite as easy as the acid alcoholysis of Erlenmeyer, and should present decided advantages if esters of rarer alcohols were to be made.

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## THE METABOLISM OF THE PHOSPHOLIPIDS

### IV. THE RATE OF PHOSPHOLIPID METABOLISM WITH SPECIAL REFERENCE TO THE QUESTION OF THE INTERMEDIARY RÔLE OF THE PHOSPHOLIPIDS IN FAT METABOLISM

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#### INTRODUCTION

The current views concerning the function of the tissue phospholipids may be quite sharply divided into two groups. On the one hand, there is the view that fat is transformed into phospholipid as a means of conveying the energy-rich fatty acids to the site of combustion within the tissue cells or, in other words, that tissue phospholipids are intermediary products in fat metabolism. On the other hand, there are the various views which ascribe to the phospholipids some essential rôle either in the structural make-up of the cell, particularly the cell membrane, or in the numerous vital processes which may be summed up under the term of general physiological activity (Bloor, 1928).

The hypothesis that the tissue phospholipids serve as a conveying mechanism in the combustion of fat seems to have been based by its originator Loew (1891) on the fact that lecithin, which differs from neutral fat only in having one of the three fatty acid radicals replaced by the phosphoric acid-choline complex, is miscible with water and is quite easily hydrolyzed in weakly acid solution. Some years later additional support was given by the observations that the level of phospholipid in the blood increased during alimentary lipemia (Bloor, 1916) and that the composition of the phospholipids in the liver is markedly influenced by the nature of the food fat (Joannovics and Pick, 1910).

In 1930 the author (1930, a) presented data to show that not only in the liver but in various other tissues the composition of

the phospholipids, as indicated by the degree of unsaturation of the constituent fatty acids, is influenced by the character of the fat in the diet. On the face of it, this observation seemed to afford further evidence that the tissue phospholipids are intermediary products in fat metabolism. It was recognized, however, that the observed influence of food fat on the phospholipids could be explained equally well by the assumption that the latter, as essential constituents of protoplasm, are subject to wear and tear, continuous repair being effected at the expense of food fat. The essential difference between these two explanations is in the *rate* of the turnover which is assumed to be going on in the tissue phospholipids. If the phospholipids are an intermediary stage in the metabolism of fat, the turnover must be quite rapid; if, on the other hand, the turnover is due to wear and tear, its rate presumably is relatively slow. The measurement of the rate of phospholipid turnover or metabolism seemed therefore to offer a possible means of obtaining information as to the function of these interesting tissue constituents. Chiefly with this purpose in mind, an extensive series of experiments was carried out in order to determine the influence of both the amount and character of the food fat on the degree of unsaturation of the phospholipid fatty acids in the tissues of the rat. The data concerning the comparative influence of various fats on the type of phospholipid synthesized by growing rats have already been published (Sinclair, 1931). It is the purpose of this paper to present the results of experiments dealing with the rate of phospholipid metabolism and to discuss the bearing of these results on the question of the function of the tissue phospholipids.

#### *Measurement of Rate of Phospholipid Metabolism*

As a result of the experiments previously described (Sinclair, 1931) it was known that the phospholipid fatty acids in both the entire body and the carcass of a rat which has been raised on an essentially fat-free, synthetic diet have a comparatively low degree of unsaturation as compared with those of a rat fed on a similar diet containing such a fat as cod liver oil. For instance, the phospholipid fatty acids of the carcass have an average iodine number of 101 in rats raised on the fat-poor diet, and 160 in rats raised on a diet containing 20 per cent of cod liver oil. Now, on

the basis of the hypothesis that the tissue phospholipids are intermediary products in fat metabolism, the values of 101 and 160 must be regarded as the average I.N. of the synthetic and cod liver oil fatty acids, respectively, while in the phospholipid stage *en route* to combustion. Furthermore, if rats are transferred from the fat-free diet on which they have been raised to the cod liver oil diet, the resulting combustion of cod liver oil fatty acids should quickly displace the synthetic fatty acids from the phospholipid molecules and thereby cause a rapid increase in the I.N. of the phospholipid fatty acids from 101 to 160. If, on the other hand, the observed influence of food fat on the composition of the tissue phospholipids is due to the utilization of ingested fatty acids both for the synthesis of new phospholipid and for the repair of wear and tear, then the transfer of rats from the fat-poor to the cod liver oil diet should be followed by a much more gradual increase in the I.N. from 101 to 160. The measurement of the rate of change in the I.N. of the phospholipid fatty acids following the transfer of rats from the fat-poor to the cod liver oil diet was therefore adopted as a means of measuring the rate of phospholipid metabolism.

*Procedure*—Young rats of both sexes were raised on a "fat-free" diet (Diet 20)<sup>1</sup> until growth had practically ceased.<sup>2</sup> A group, selected as to age and weight, was then transferred to the cod liver oil diet (Diet 260) and, at suitable intervals, individuals were removed and a determination made of the amount and I.N. of the phospholipid fatty acids either in the entire animal or in the carcass.<sup>3</sup> The procedure employed in extracting, purifying, and saponifying the phospholipids has been given in detail in an earlier paper (Sinclair, 1930, c).

*Results*—In Fig. 1 is presented the result of our first measure-

<sup>1</sup> The numbers refer to the diets listed in Table I (Sinclair, 1931).

<sup>2</sup> Neither the purified fat-free diet (Diet 20) nor the unpurified fat-poor diet (Diet 1 or 3) is adequate for normal growth. However, except for their subnormal size, rats raised on these diets are quite normal in appearance and behavior, provided they are kept in the ordinary stock cage. If kept in false bottom cages, the rats develop scaliness of the feet and tail (Sinclair, 1930, d).

<sup>3</sup> The carcass comprises the bony skeleton and its musculature. The phospholipids of the carcass are chiefly those contained in the skeletal muscle.

ment of the rate of phospholipid metabolism. The data show the rate of increase in the I.N. of the phospholipid fatty acids in the carcasses of rats following transfer from the customary fat-free diet to one containing cod liver oil.<sup>4</sup> A similar, rapid increase in the I.N. of the phospholipid fatty acids in the entire bodies of rats was observed. On the 1st, 2nd, 3rd, 6th, 9th, and 13th days after transfer from the fat-free diet to one containing cod liver oil, the I.N. was 115, 125 and 129, 128 and 131, 137, 138, and 146, respectively, the I.N. of the phospholipid fatty acids in the entire bodies of the controls being  $101 \pm 4.2$ .

It is obvious from Fig. 1 that the rate of turnover of the phospholipids in the tissues of the rat is quite remarkable. Bearing

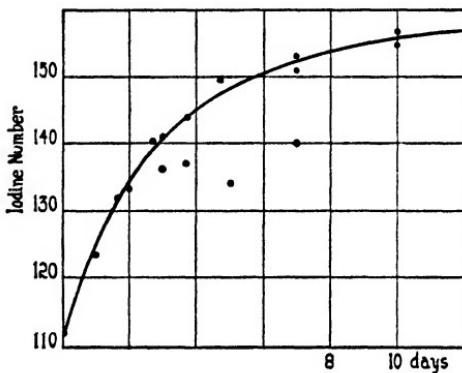


FIG. 1. The increase in the degree of unsaturation of the phospholipid fatty acids in the carcass of the rat following transfer from the fat-free to the 20 per cent cod liver oil diet.

in mind that the average I.N. of the phospholipid fatty acids in the entire bodies and in the carcasses of rats raised on the cod liver oil diet are 146 and 160, respectively, one sees that within 2 weeks there is a practically complete replacement of the original more

<sup>4</sup> It will be observed that the phospholipid fatty acids in the carcasses of the control rats in these first experiments had an average I.N. of 112; i.e., considerably higher than the I.N. value of 101 found to be characteristic of the fat-poor diet. It is believed that this difference is due largely to the fact that in these earlier experiments the rats were somewhat larger when started on the fat-free diet, and were kept on the diet for a shorter period than the rats used in later experiments.

saturated fatty acids by the more highly unsaturated acids of the ingested cod liver oil. It is evident also that the replacement process, in the entire body of the rat, is approximately 30 per cent complete at the end of the 1st day on the cod liver oil diet. This means of course that the actual turnover of phospholipid in the entire rat amounts to somewhat more than 30 per cent per day, the change in I.N. becoming progressively less and less as the amount of original phospholipid diminishes. Since a daily turnover of this order of magnitude seemed quite incompatible with our usual conception of the probable rate of protoplasmic wear and tear, it was concluded that part, at least, of the tissue phospholipids is probably involved as an intermediary product in the metabolism of fat (Sinclair, 1930, b).

*Does a Relationship Exist between Rate of Turnover of Phospholipids and Intensity of Fat Metabolism?*

It is an obvious deduction from the hypothesis that fatty acids of ingested fat pass through the stage of tissue phospholipid *en route* to combustion that since the amount of phospholipid remains constant, the rate of turnover of the phospholipids should be directly proportional to the intensity of fat metabolism.

According to the observations of Benedict and MacLeod (1929) the metabolism of the rat is inversely proportional to the environmental temperature, the metabolism at 12° being roughly double that at 28°. This being the case, the food consumption should be materially increased by exposure of rats to low environmental temperatures. Accordingly, if the rate of turnover of the phospholipids is directly proportional to the amount of fat being burned, then the rate of turnover should be appreciably greater in rats kept at about 14° than it is in rats kept at 28°. This deduction was the basis for the following experiments.

The procedure employed was in general the same as that just described. From a fairly large number of rats which had been raised on our fat-poor diets (Diets 1 and 3) for about 3 months, pairs of about the same age and weight were selected. One of the pair was placed in a room kept at between 28-30°, the other in a room kept at about 14-15°.<sup>5</sup> After a preliminary period of several

<sup>5</sup> The author is indebted to Dr. J. R. Murlin for permission to use the thermoregulated room of the Department of Vital Economics.

days, during which their customary fat-poor diet was fed, the rats were transferred to the 21.3 per cent cod liver oil diet (Diet 262).

**Results**—The results of our first determination of the possible influence of environmental temperature, and therefore of the intensity of fat metabolism, on the rate of turnover of the phospholipids are given in Table I. On comparing the values given in Columns 4, 7, and 10, one observes at once that the rate of increase in the I.N. of the phospholipid fatty acids in the carcasses was no more rapid in rats which were kept at such low temperatures as 14° and 5° than it was in those rats kept at 28°. The turnover in

TABLE I  
*Lack of Influence of Environmental Temperature on Rate of Phospholipid Metabolism*

Time on diet (1)	Room temperature 28-30°			Room temperature 14°			Room temperature 5°		
	Body weight		I.N. of phospholipid fatty acids (4)	Body weight		I.N. of phospholipid fatty acids (7)	Body weight		I.N. of phospholipid fatty acids (10)
	Initial (2)	Final (3)	gm.	Initial (5)	Final (6)	gm.	Initial (8)	Final (9)	gm.
days									
1	202	202	110	172*	167	115	132*	129	115
2	215	220	126	191	190	125	132	128	123
2	195	193	123						
3	212	223	129	186	185	132	158	152	130
3	190	190	127						
5	207	221	134	175	183	137			

\* With these two exceptions all the rats used were males.

the tissue phospholipids did occur, but apparently its rate was quite independent of the intensity of the fat metabolism.

It was believed that the importance of the problem warranted another experiment along the same lines. In this second experiment, the results of which are given in Table II, a record was kept of the daily food consumption in order to have direct proof that the rats at the lower temperature ate more, and therefore burned more fat than those at the higher temperature. Also, in this second experiment, determinations were made of the I.N. of the phospholipid fatty acids in the livers as well as in the carcasses. The results of this second experiment are in complete agreement with those of the first in showing that there is no relationship be-

tween the rate of turnover of the phospholipids and the intensity of fat metabolism.

There are a few points brought out by the data in Tables I and II which should be commented upon at this time. In the first place, it will be observed that many of the rats gained appreciably in weight during the few days on the cod liver oil diet, although they had maintained a practically constant weight for some time before the change in the diet. This resumption of growth on

TABLE II

*Lack of Influence of Food Consumption on Rate of Phospholipid Metabolism*

Time on diet	Room temperature 10-11°					Room temperature 28-30°				
	Body weight		Food consumed	I.N. of phospholipid fatty acids		Body weight		Food consumed	I.N. of phospholipid fatty acids	
	Initial	Final		Carcass	Liver	Initial	Final		Carcass	Liver
Control rats left on the unpurified fat-poor Diet 3										
days	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
6	142	126	59.5	101	124	128	124	38.0	103	120
Rats changed from Diet 3 to Diet 262 containing 21.3 per cent cod liver oil										
1	118	110		109		118	122	10.3*	111	176
2	128	124	6.8	123		126	131	10.4	126	175
2	138	134	24.2*	124	192	132	134	13.5*	120	187
3	146	150	20.3	133	190	162	164	12.9	132	188
5	132	140	53.5	136	182	130	142	32.0	139	181
7	156	153	66.8	142	176	158	174	52.9	144	179

All rats used in these experiments were females.

\* With these exceptions the data on the food consumption for 1 day are lacking.

changing from the fat-poor to the fat-rich diet is in line with the observations of Burr and Burr (1929, 1930), McAmis, Anderson, and Mendel (1929), and Evans and Lepkovsky (1931) that fat *per se* has growth-promoting properties. Since the greater part of the change in the composition of the phospholipids occurred before the gain in weight had become significant, it is evident that there is no relationship between the turnover in the phospholipids and the formation of new phospholipid due to growth.

On comparing the changes in the composition of the liver phos-

pholipids with those of the carcass, it is evident that the turnover in the phospholipids of the liver is much more rapid than it is in the carcass. In the former the replacement of the more saturated fatty acids originally present by the more highly unsaturated acids characteristic of the cod liver oil diet is practically complete within 2 days. This rapid turnover in liver phospholipids on feeding cod liver oil is in agreement with the observations of Joannovics and Pick (1910) and of the author (1929).

*Rate of Turnover of Phospholipids during Fasting, Etc.*

As a result of experiments which will be published in a subsequent paper, it was known that the addition of 1 per cent cod liver oil to an otherwise purified fat-free diet results in the synthesis by rats raised on such a diet, of tissue phospholipids with an astonishingly high degree of unsaturation, whereas the depot fat has the characteristic I.N. of synthetic fat. On the basis of the hypothesis that the tissue phospholipids are intermediary products in fat metabolism, the I.N. of about 140 in the phospholipid fatty acids in the carcasses of rats raised on the 1 per cent cod liver oil diet must indicate the simultaneous combustion of the ingested cod liver oil and some synthetic fat. If the explanation is correct, then the exclusive combustion of synthetic fat during fasting should cause a rapid decrease in the I.N. from about 140 to about 100. Furthermore, since the average I.N. of the phospholipid fatty acids in rats raised on a 20 per cent coconut oil diet is 124 (Sinclair, 1931), the feeding of coconut oil to rats raised on the 1 per cent cod liver oil diet should cause the I.N. to fall rapidly from about 140 to about 124.

The results of experiments which have been carried out along these lines are given in Table III. The effect of fasting on the composition of the phospholipids is shown by the data in Experiment 2. It is evident that while a decrease in the I.N. of the phospholipid fatty acids had occurred, the I.N. even at the end of 6 days of fasting was still such as to indicate the presence of the more highly unsaturated acids of the previously ingested cod liver oil. Similarly the data given in Experiment 4 show that even after 6 days on the coconut oil diet there is no evidence of any displacement from the phospholipid molecules of the original highly unsaturated fatty acids by the more saturated fatty acids of the coconut oil.

In Experiment 3 four rats were transferred from their customary 1 per cent cod liver oil diet to the purified fat-free diet (Diet 20). The data show that although the values for the I.N. are somewhat lower than in the controls, nevertheless there has been a very slight reduction, even after several weeks, towards the value of 100 characteristic of the fat-free diet.

The various experiments of this series indicate not only that food fatty acids are not incorporated into tissue phospholipid as a stage

TABLE III  
*Influence of Various Experimental Conditions on Iodine Numbers of Phospholipid Fatty Acids in Carcasses of Rats Raised on Diet Containing 1 Per Cent Cod Liver Oil*

	Duration	Body weight		I.N. of phospholipid fatty acids	I.N. of acetone-soluble lipids
		Initial	Final		
	days	gm.	gm.		
Experiment 1, controls				143	61
				171	60
				153	63
				146	59
Experiment 2, fasting	2	187	168	131	60
	4	191	154	129	61
	6	188	145	125	65
Experiment 3; changed to diet practically fat-free (Diet 20)	4	202	207	134	59
	8	211	214	131	59
	14	208	210	135	61
	21	191	194	129	62
Experiment 4; changed to Diet 262 containing 19.3 per cent coconut oil	2	147	151	143	61
	3	142	147	140	61
	6	145	153	137	54

in combustion, but also that the wear and tear of tissue phospholipid is probably a very slow process.

#### *Comparative Change in Composition of Phospholipids Induced by Various Fats*

In the light of the results obtained in the various experiments just described it became quite evident that the rapid change which occurred in the composition of the phospholipids of rats

after transfer from their customary fat-poor diet to one containing cod liver oil was probably due neither to the involvement of the phospholipids as intermediaries in the metabolism of fat nor to

TABLE IV

*Rate of Change in Degree of Unsaturation of Phospholipid Fatty Acids in Carcass and Liver Following Change in Diet*

Time on diet	Body weight		I.N. of phospholipid fatty acids		Time on diet	Body weight		I.N. of phospho- lipid fatty acids	
	Initial	Final	Carcass	Liver		Initial	Final	Carcass	Liver
<b>Control rats raised on unpurified Diet 3</b>									
days	gm.	gm.				99±3.6*	119±3.1†		
<b>Transferred from Diet 3 to Diet 262, coconut oil</b>									
1	126	128	114	163	days	gm.	gm.		
2	126	128	125	171	7	161	104		
3	132	144	128	181	7	146	156	103	137
5	146	162	138	184	14	144	148	103	134
14	124	144	141	174	29	152	152	107	143
14	122	140	148	183	56	136	148	110	140
21	140	160	148	178	88	136	142	114	139
35	160	210	155	177	<b>Raised on coco- nut oil diet</b>				
66	136	170	156	174		124	143		
<b>Raised on cod liver oil diet</b>			160	181	<b>Transferred from Diet 262, cod liver oil, to Diet 262, coconut oil</b>				
3	176	180	117	169	7	182	192	151	
3	166	166	112	158	7	168	170	153	178
5	158	168	127	158	14	168	170	151	167
6	152	150	114	159	21	154	164	149	165
7	146	162	127	173	<b>Transferred from Diet 3 to Diet 262, olive oil</b>				
<b>Raised on lin- seed oil diet</b>			137	156	3	130	138	101	146
3	176	180	117	169	4	162	168	112	161
3	166	166	112	158	5	170	180	105	147
5	158	168	127	158	7	128	146	108	152
6	152	150	114	159	7	150	156	113	159
7	146	162	127	173	14	130	132	127	159
<b>Raised on lin- seed oil diet</b>					<b>Raised on olive oil diet</b>				
								134	151

\* Average of fifteen determinations.

† Average of eight determinations.

wear and tear. It became necessary therefore to cast about in new directions for clues as to the true significance of phospholipid me-

tabolism or turnover. To this end, it was decided to see whether a change occurred in the composition of the phospholipids of rats when fed such fats as linseed oil, olive oil, and coconut oil.

The results obtained are given in Table IV. The significant facts borne out by these data are as follows: (1) The ingestion of linseed, olive, and coconut oils by rats which have been raised on the fat-poor diet does cause an increase in the degree of unsaturation of the phospholipid fatty acids in both the livers and carcasses. (2) The change in the composition of the phospholipids is much more rapid in the liver than in the carcass. (3) The rate of the change induced in the phospholipids of the carcass by these various oils differs markedly from one oil to another, the rate decreasing in the following order: cod liver oil, linseed oil, olive oil, and coconut oil. The change induced by coconut oil is very slow indeed, being only about 58 per cent complete after a period of 12 weeks; with cod liver oil the change in I.N. is about 65 per cent complete in 5 days.

The results obtained in the experiments in the preceding section had indicated that if the phospholipids already have a high degree of unsaturation, they give up their unsaturated fatty acids very slowly. This conclusion is still further borne out by the results of an experiment in which four rats were transferred from the cod liver oil diet on which they had been raised, to a diet containing the same amount of coconut oil. The results of this experiment are given in Table IV. It is evident that even after 3 weeks the I.N. of the phospholipid fatty acids in both the carcass and the liver is still considerably higher than the values characteristic of the coconut oil diet (I.N. 124 in the carcass and 143 in the liver).

#### DISCUSSION

The most significant facts brought out by this study of phospholipid metabolism may be said to be: (1) The ingestion of fat such as cod liver oil by rats which have been raised on a fat-free or fat-poor diet results in a marked increase in the degree of unsaturation of the phospholipids in skeletal muscle and in the liver. (2) Since the amount remains constant,<sup>6</sup> a change in the degree of

<sup>6</sup> That the amount of phospholipid in the carcasses of rats does not change following transfer from their customary fat-poor diet to one containing about 20 per cent of fat is quite definitely shown by the following data. The

unsaturation must indicate a change in the composition as regards the constituent fatty acids. (3) The rate of increase in the I.N. of the phospholipid fatty acids following the ingestion of cod liver oil is quite rapid; the turnover in the liver phospholipid is 100 per cent complete, and in the carcass phospholipids about 50 per cent complete within 3 days. (4) The rate of turnover in the phospholipids of the carcass is not proportional to the intensity of fat metabolism. (5) In sharp contrast to the ease with which the more saturated fatty acids are replaced by more highly unsaturated acids, the latter are apparently retained within the phospholipid molecule with great tenacity and for a relatively long period of time.

In the light of these facts the following conclusions seem justified: first, that the phospholipids of the muscles, at any rate, are not involved as intermediary products in the metabolism of fat; second, that the influence of food fat on the composition of the phospholipids of animal tissues is not due, entirely at any rate, to the utilization of ingested fatty acids in the repair of phospholipid which has been broken down as a result of the wear and tear of protoplasm.

What then is the function of the tissue phospholipids, and what is the explanation of the readiness with which they attain and the tenacity with which they maintain a high degree of unsatura-

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average amount of phospholipid fatty acids in the carcasses of fifteen rats raised on the unpurified diets was 0.954 gm. per 100 gm. of moist fat-free tissue (high, 1.049; low, 0.903) and 3.50 gm. per 100 gm. of dry extracted tissue (high, 3.92; low, 3.26). In the carcasses of forty rats raised on the unpurified diets and transferred to the fat-rich diet from 1 to 88 days before death, the average amount of phospholipid fatty acids was 0.958 gm. per 100 gm. of moist fat-free tissue (high, 1.052; low, 0.886) and 3.63 gm. per 100 gm. of dry extracted tissue (high, 4.11; low, 3.39). On the other hand, there is some reason for believing that the amount of phospholipid in the *liver* may increase somewhat following the transfer from the fat-poor to the fat-rich diet. The average amount of phospholipid fatty acids in the livers of eleven rats raised on the unpurified diets was 2.36 gm. per 100 gm. of moist fat-free tissue (high, 2.73; low, 2.19) and 9.34 gm. per 100 gm. of dry extracted tissue (high, 11.55; low, 8.00). In the livers of forty rats transferred from the unpurified to the fat-rich diet from 1 to 88 days before death, the average amount of phospholipid fatty acids was 2.61 gm. per 100 gm. of moist fat-free tissue (high, 3.32; low, 2.18) and 10.22 gm. per 100 gm. of dry extracted tissue (high, 16.28; low, 7.76).

tion? Until more information has been obtained concerning the factors involved in bringing about changes in the composition of the phospholipids, it is thought to be unwise to attempt to answer these questions. Further study of the problem is in progress.

There still remain a few points connected with the results given above which warrant further discussion. It has already been pointed out that all rats which have been raised on either the fat-free or the fat-poor diet have ceased to grow when still quite subnormal in weight. They have been, however, entirely free from any other signs of abnormality. The question arose as to whether or not the rapid turnover in the composition of the phospholipids following the ingestion of a fat such as cod liver oil or linseed oil was due to some abnormality other than the low degree of unsaturation. To test this point, rats which had been raised on diets containing such fats as coconut oil, olive oil, lard, and linseed oil, and which had grown normally, were fed on the cod liver oil diet for 3 days. In these rats the I.N. of the phospholipid fatty acids was found to be considerably higher than the values characteristic of the fat diet on which they had been raised. It is evident therefore that the occurrence of an increase in the degree of unsaturation of the tissue phospholipids following the ingestion of fat depends entirely upon the nature of the fat and the initial degree of unsaturation of the phospholipids.

On comparing Figs. 1 and 2 one observes that the rate of increase in the I.N. of the phospholipid fatty acids is considerably greater in the former than in the latter. For instance, in the earlier experiments I.N.'s of 155 and 157 were obtained on the 10th day after transfer to the cod liver oil diet, whereas in the later experiments the I.N. never exceeded 150 within 3 weeks. It was thought that this difference in the rate of turnover of the phospholipids might have been due to the fact that the rats used in the first experiments were younger and smaller than those used later. To test this point the rate of increase in the I.N. was measured in rats which had been on the unpurified diet for only about 5 weeks and were still actively growing. The I.N. obtained on the 1st, 2nd, 3rd, and 5th days after transfer to the cod liver oil fell within the range of values given in Fig. 2. No explanation for the above difference has as yet been obtained.

In Fig. 2 are plotted the I.N.'s obtained in a few rats after

transfer from the fat-poor diet to diets containing either 4.1 or 8.7 per cent instead of the usual 21.3 per cent of cod liver oil. These few results indicate that there is a relationship between the rate of turnover in the tissue phospholipids and the percentage of fat in the diet. At first sight this conclusion would seem to contradict the previous conclusion that there is no relationship between the rate of turnover and the intensity of fat metabolism. The two observations can be brought into harmony if it is assumed that the rate of increase in the I.N. is a function of the concentration

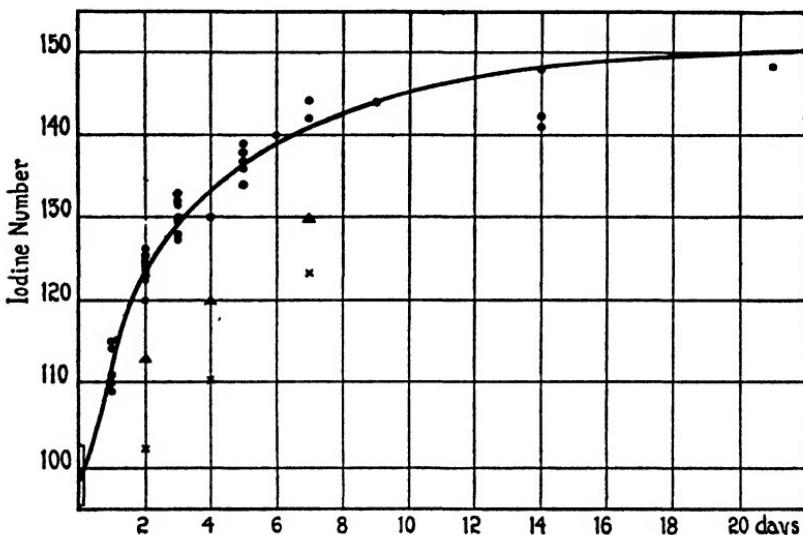


FIG. 2. Showing that the rate of increase in the I.N. of the phospholipid fatty acids is influenced by the percentage of cod liver oil in the diet. The points on the chart indicate the I.N. of the phospholipid fatty acids in the carcasses of rats at various times after transfer from the unpurified diet on which they had been raised to a diet containing either 21.3 per cent (●), 8.7 per cent (▲), or 4.1 per cent (×) of cod liver oil.

of highly unsaturated fatty acids within the tissues. In those experiments in which the amount of fat burned per day was changed by altering the environmental temperature, the concentration of fat in the tissues probably remained about the same, although the amount of fat which passed into the tissue cells was greater at the lower temperature than at the higher. On the other hand, under

normal conditions the concentration of fat in the tissues is probably a function of the percentage of fat in the diet.

#### SUMMARY AND CONCLUSIONS

The rate of metabolism or turnover of the tissue phospholipids has been measured by determining the rate of change in the degree of unsaturation of the phospholipid fatty acids in the tissues of the rat following transfer from one distinctive diet to another. Previous work had shown that for each distinctive diet there is a characteristic degree of unsaturation of the tissue phospholipids.

It has been found that following the transfer of rats from the customary fat-poor diet to one containing cod liver oil there is a rapid increase in the iodine number of the phospholipid fatty acids in the entire body, the carcass (*i.e.* chiefly the skeletal muscles), and the liver. Since there is no change in the amount of phospholipid present in the tissues, this increase in unsaturation has been taken as evidence of a change in the composition of the phospholipids as regards the constituent fatty acids. The rate of turnover in the composition of the phospholipids is considerably more rapid in the liver than in the skeletal muscles.

An increase in the iodine number of the phospholipid fatty acids also occurs following transfer from the fat-poor diet to one containing linseed oil, olive oil, or coconut oil. The rate of increase, however, is markedly different from one fat to another; it is rapid with cod liver and linseed oils, intermediate with olive oil, and very slow with coconut oil.

The rate of turnover of the phospholipids in the skeletal muscles after the ingestion of a standard cod liver oil diet was found to be constant, irrespective of the intensity of fat metabolism.

It was found that in contrast to the rapidity with which the degree of unsaturation of the phospholipids increased from a lower level to a higher, the decrease from a higher to a lower level of unsaturation was an extremely slow process.

The most significant conclusions resulting from this work are: first, that the phospholipids present in the muscles and presumably those in the other tissues as well, should no longer be regarded as intermediary products in the metabolism of fat; second, that food fat may exert an influence on the degree of unsaturation of the tissue phospholipids which is quite apart from that which may

be due to the utilization of food fat for the repair of wear and tear.

The tissue phospholipids apparently have a marked tendency to attain and having once attained, to maintain a high degree of unsaturation.

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## CATALYTIC OXIDATION OF THE CARBOHYDRATES AND RELATED COMPOUNDS BY OXYGEN IN THE PRESENCE OF IRON PYROPHOSPHATES\*

### II. METHYL ALCOHOL, FORMALDEHYDE, FORMIC ACID, AND SODIUM FORMATE

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Tables giving the amount of carbon dioxide obtained from the oxidation of *d*-glucose and related sugars by oxygen in the presence of iron pyrophosphates, appeared in a previous paper.<sup>1</sup> Though no data were submitted, the statement was made that sodium formate is not oxidized to carbon dioxide under similar conditions. This conclusion was based upon short preliminary experiments by the authors. However, Spoehr and Smith<sup>2</sup> had reported that "A portion of this acid (sodium salt) was immediately oxidized, but the formation of carbon dioxide soon ceased. The oxidation of glucose is possible whether the ferro or ferri compound is initially present. With formic acid, however, as soon as the ferro compound is oxidized to the ferri form, the oxidation proceeds no further. Formic acid does not reduce the ferric compound, or only very slowly,<sup>3</sup> and thus cannot regenerate the ferrous compound which is the active agent in the oxidation . . . ." Obviously some doubt remained as to the result of a prolonged oxidation of sodium formate.

Since the oxidation of the simple sugars by oxygen in the presence of iron pyrophosphates is being continued to determine, if

\* This is a continuation of the work begun at the University of Nebraska at the suggestion of Dr. H. A. Spoehr, and under the supervision of Dr. F. W. Upson. The first article of this series appeared in *J. Biol. Chem.*, **94**, 423 (1931).

<sup>1</sup> Degering, E. F., and Upson, F. W., *J. Biol. Chem.*, **94**, 426 (1931).

<sup>2</sup> Spoehr, H. A., and Smith, J. H. C., *J. Am. Chem. Soc.*, **48**, 241 (1926).

<sup>3</sup> Italicized by the author of this article.

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possible, the other end-products of these reactions, and since formic acid is formed in these experiments (alkaline solution), some definite results on the oxidation of sodium formate under identical conditions become imperative.

TABLE I  
*Carbon Dioxide Production from Sodium Formate and Formaldehyde*

Days.....		1	2	5	8	12	16
5.74 gm. sodium formate	CO <sub>2</sub> , gm.....	0.0009	0.0017	0.0024	0.0013	0.0024	0.0022
	C returned as CO <sub>2</sub> , per cent..	0.02	0.05	0.07	0.04	0.07	0.06
	No. of deter- minations.....	18	16	14	12	10	8
13.62 gm. sodium formate	CO <sub>2</sub> , gm.....	0.000	0.0003	0.0021	0.0029	0.0009	0.0013
	C returned as CO <sub>2</sub> , per cent..	0.00	0.00	0.02	0.02	0.01	0.01
	No. of deter- minations.....	9	8	7	6	5	4
2.53 gm. formal- dehyd e	CO <sub>2</sub> , gm.....	0.0008	0.0017	0.0030	0.0029	0.0038	0.0037
	C returned as CO <sub>2</sub> , per cent..	0.02	0.05	0.09	0.09	0.1	0.1
	No. of deter- minations.....	18	16	14	12	10	8
6.0 gm. formal- dehyd e	CO <sub>2</sub> , gm.....	0.0002	0.0002	0.0022	0.0026	0.0023	0.0025
	C returned as CO <sub>2</sub> , per cent..	0.00	0.00	0.02	0.02	0.02	0.02
	No. of deter- minations.....	9	8	7	6	5	4

## EXPERIMENTAL

The procedure followed was identical with that reported in the previous paper.<sup>4</sup> Sodium formate, formic acid, formaldehyde, and methyl alcohol were studied. The data for sodium formate and formaldehyde are given in Table I; those for sodium formate and formic acid are given in Table II. Since methyl alcohol is the most stable of this group, data for methyl alcohol seem unnecessary.

<sup>4</sup> Degering, E. F., and Upson, F. W., *J. Biol. Chem.*, **94**, 424 (1931).

It was thought that the disodium phosphate used as a buffer might hold an appreciable amount of carbon dioxide in solution, and thus obscure the results. To check this, the solutions in one series of experiments were acidified at the end of 16 days with  $H_3PO_4$ , and the determinations continued for 4 days more. These results, representing a 20 day determination, appear in Table II.

#### DISCUSSION

From Tables I and II it is seen that the maximum value for carbon returned as carbon dioxide is only 0.3 per cent at the end of a 20 day determination. The corresponding value for the four controls is 0.2 per cent (calculated on the same basis). If this correction be applied, only 0.1 per cent of the carbon was returned

TABLE II  
*Total CO<sub>2</sub> from Tubes Acidified with H<sub>3</sub>PO<sub>4</sub> after 16 Days*

Compound used.....	HCOONa	Controls	HCOOH
CO <sub>2</sub> for 20 days, gm.....	0.0122	0.0086	0.0041
C returned as CO <sub>2</sub> , per cent.....	0.3	0.2	0.1
No. of determinations.....	3	4	3

as carbon dioxide at the end of the 20 day determination. Since this represents the maximum value for the per cent of carbon returned as carbon dioxide, and since this value is well within the range of experimental error for the procedure used, there is no indication that oxidation occurred. Titrations for acid production in the experiments with formaldehyde and permanganate titrations for total reducing constituents in the experiments with sodium formate and formic acid lead to the same conclusions.

#### SUMMARY

In conclusion, it appears (1) that there is no formation of carbon dioxide from methyl alcohol, formaldehyde, formic acid, or sodium formate; (2) that there is no indication of the oxidation of formaldehyde to formic acid; (3) that these data present additional proof of the correctness of Spoehr's conclusion that formic acid does not reduce the ferri form (which is the form used in this series of ex-

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periments on the oxidation of the simple sugars) of the catalyst to the ferro form; and (4) that there is no apparent reason why formic acid cannot be determined quantitatively as an end-product in the oxidation of the simple sugars by oxygen in the presence of iron pyrophosphates under the conditions of these experiments.

## GLYCOGENESIS FROM GLUCOSE ADMINISTERED TO THE FASTING DOG

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### INTRODUCTION

A complete, though temporary, loss of the ability to burn glucose was observed in dogs after a fast of 3 weeks or more with frequent exercise on the treadmill (1). During the first 4 hours after 50 gm. of glucose were ingested, the blood sugar rose to a high level and part of the sugar was excreted, but at least 30 gm. could not be accounted for either by excretion or oxidation. The fate of this fraction involves the question of whether or not the failure of oxidation is accompanied by an impairment in the process of storage of glycogen.

In a series of experiments by Junkersdorf and Mischnat (2), in which a fasting period of 11 days was employed, there was apparently no increase in glycogen in the liver and muscles after giving 3 gm. of glucose per kilo, for the livers contained, in four fasting dogs, an average of 0.90 per cent, and, in eleven dogs killed 2 to 10 hours after glucose ingestion, an average of 0.92 per cent; the corresponding figures for muscle were 0.21 and 0.20 per cent, respectively. No increase, on the average, was observed in the heart, while a small increase was found in the wall of the gastrointestinal tract after 2 hours.

In contrast to these observations Cori (3) found that in rats fasted for 48 hours the ingestion of a diet rich in carbohydrates produced an increase in liver glycogen of nearly 4 gm. per 100 gm. of liver. Barbour, Chaikoff, Macleod, and Orr (4) obtained very similar results and also found that the muscle glycogen rose from 0.25 to 0.36 per cent.

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As a step in the investigation of the problem of carbohydrate metabolism in "hunger diabetes," determinations were made of the amount of glycogen deposited in the tissues, especially the liver and muscles, of dogs which had ingested glucose after a fast of at least 4 weeks. In order to obtain a more complete picture of the carbohydrate changes than could be derived from glycogen determinations alone, and as a check on glycolysis during the handling of the tissues, analyses were also made for other carbohydrates and for lactates.

#### *Methods*

Three mongrel dogs, one female and two males, were used for the experiments. The procedure here described for Dog 51 (Table I) is essentially the same as that employed with the other animals. During 17 days of fasting the dog was exercised on the treadmill for nine half hour periods. On the 20th day, under amyntal anesthesia, the semitendinosus muscle of the right leg was completely exposed. The blood vessels were then clamped and the whole muscle quickly removed by a single cut across each end and through the blood vessels. The following day the function of the operated leg seemed unimpaired. Amyntal was given again on the 22nd day of fast and the liver exposed by a mid-line incision. Two pieces of liver, about 7 gm. each, were excised by electric cautery, one from the lower border of the left lobe, the other from the vesicular lobe. The other semitendinosus muscle (left leg) was then completely removed. Blood was drawn from the heart on the 22nd day and again on the 27th day. The glucose was given on the 28th day. To make the conditions for absorption and utilization of the sugar comparable to those of the calorimeter experiments (1), approximately 4 hours were allowed to elapse before the anesthetic was injected. The procedure for the 28th day is tabulated in the following protocol.

- 9.36 a. m., ingested 25 gm. glucose
- 11.36    "    blood from heart
- 1.25 p. m., amyntal anesthesia
- 1.43    "    blood from heart
- 1.52    "    excised tissue from margin of right lobe of liver
- 1.55    "       "    "    "    central    "    "    "
- 2.06    "       semimembranosus muscle of right leg

- 2.10 p. m., excised right side of diaphragm
- 2.11 " " left " " "
- 2.12 " " apex of heart, rhythmically contracting
- 2.13 " " section from middle of left ventricle

The remainder of the liver was removed and weighed. The weight (sum of the parts excised after glucose ingestion) is given in Table II. The urine was collected from the bladder and the alimentary canal was then removed. Glucose was determined in the contents by Benedict's method after precipitation of the proteins by heating to boiling with acetic acid.

The variations as to days of fast when tissue samples were taken are shown in Table II. Dogs 36 and 50 each received 50 gm. of glucose, part of which was vomited between 3 and 4 hours later.

Duplicate samples of tissue of 3 to 5 gm. each were rapidly prepared for carbohydrate analyses according to a method embodying suggestions by Evans (5). The muscle was divided longitudinally and each portion weighed on a torsion balance to within 0.1 gm. and immersed in 30 cc. of 75 per cent alcohol at  $-5^{\circ}$  in a mortar surrounded by an ice-salt mixture. Liver samples were similarly treated after discarding the cauterized portion. Usually about 1 minute, and in no case more than  $2\frac{1}{2}$  minutes, elapsed between the excision of the tissue and its transfer to the alcohol. Each sample was minced with scissors, ground with a pestle, and rinsed with 10 cc. more of 75 per cent alcohol into a flask and kept at  $-5^{\circ}$  to  $-8^{\circ}$  for about 24 hours. It was then filtered through paper and washed with several 5 cc. portions of 75 per cent alcohol.

The filtrate was used for the determination of lactic acid and glucose, as described later, and the extracted tissue residue was analyzed for glycogen as follows:<sup>1</sup> The residue was pressed with a porcelain spatula to remove alcohol and transferred to a tube containing sufficient hot 60 per cent KOH to make 1 cc. per gm. of original tissue. The tubes were covered with funnels and kept in a boiling water bath for 3 hours. The samples were then partially cooled, diluted to 10 or 15 cc., and 5 cc. portions transferred to centrifuge tubes, where they were nearly neutralized with concentrated HCl (litmus paper indicator). 1.5 cc. of 95 per cent

<sup>1</sup> We are indebted to Dr. Esther M. Greisheimer for several suggestions (personal communication, 1929) which we have included in this modification of Pflüger's method.

alcohol and sufficient water to make 10 cc. were added. After thorough mixing the samples were centrifuged, filtered, and a 5 cc. aliquot portion (equivalent to one-fourth or one-sixth of the original tissue) was mixed with 10 cc. of 95 per cent alcohol. The sample was left in the refrigerator overnight and the precipitated glycogen was separated by centrifugation and washed once with 95 per cent alcohol. Hydrolysis was carried out with 2.2 per cent HCl for 3 hours, after which the acid was neutralized to phenol red with 1 N NaOH. All glucose determinations were made by the Somogyi modification of the Shaffer-Hartmann method (6). A 3 per cent correction was added for loss during hydrolysis, as determined by Nerking (7).

The filtrates were treated with the Somogyi (8) protein-precipitating reagents (8 cc. of Reagent I, 1 cc. of Reagent II), made up to definite volume, shaken, and filtered. Lactic acid was determined by the Friedemann-Kendall method (9) on a 10 to 25 cc. sample, after removing alcohol by diluting with 40 cc. of water and boiling down to 10 cc. The copper-lime precipitation was omitted as unnecessary for the purpose of these experiments since it had previously been found to make little difference in some diabetic blood and tissue filtrates.

Total reducing material in the Somogyi filtrate was determined and reported as "soluble carbohydrate." The non-fermentable reducing substances included in this figure probably amounted to about 6 per cent, which was the average found in six filtrates which were subjected, after removal of alcohol, to yeast fermentation according to the method of Benedict (10). The absence of higher hydrolyzable carbohydrates was shown in two liver and two heart samples in which the average difference between unhydrolyzed and hydrolyzed samples was found to be 2 per cent.

As a typical set of results showing the agreement between duplicate pieces of tissue, complete data on Dog 51 are given in Table I; glycogen and soluble carbohydrates are calculated as glucose, and the sum of these plus lactic acid (potential glucose) is given in the columns designated "Total."

Since the experimental work described here was completed Evans, Tsai, and Young (11) have questioned the use of amyta in studies on liver glycogen. We selected amyta as preferable to the volatile or other anesthetics because the normal resting car-

TABLE I  
*Carbohydrate Content of Tissues during Fast and 4 Hours after Glucose  
 Dog 51, weight 6.1 kilos.*

	Day of fast.....			20			20			22			28		
	Glycogen.....	Soluble carbohydrates.....	Lactic acid.....	Glycogen.....	Soluble carbohydrates.....	Lactic acid.....	Glycogen.....	Soluble carbohydrates.....	Lactic acid.....	Glycogen.....	Soluble carbohydrates.....	Lactic acid.....	Glycogen.....	Soluble carbohydrates.....	Lactic acid.....
Muscle, Sample 1.....	0.260	0.038	0.021	0.319	0.262	0.042	0.023	0.327	0.338	0.054	0.052	0.444	0.291	0.039	0.020
" 2.....	0.321	0.039	0.015	0.375	0.177	0.052	0.029	0.258	0.319	0.073	0.028	0.420	0.322	0.039	0.020
Average.....	0.291	0.039	0.018	0.348	0.220	0.047	0.026	0.293	0.328	0.064	0.040	0.432	0.322	0.039	0.020
Liver, Sample 1.....				1.302	0.240	0.013	1.555	4.522	0.304	0.022	4.848				
" 2.....				0.983	0.251	0.013	1.247	4.445	0.243	0.017	4.705				
Average.....				1.143	0.245	0.013	1.401	4.484	0.274	0.020	4.777				
Heart, Sample 1.....										1.527	0.064	0.053	1.644		
" 2.....										1.851	0.057	0.064	1.972		
Average.....										1.689	0.061	0.058	1.808		
Diaphragm, Sample 1.....										0.237	0.081	0.037	0.355		
" 2.....										0.141	0.084	0.080	0.305		
Average.....										0.189	0.082	0.053	0.329		
Blood.....															
Before glucose.....										0.372	0.014				
2 hrs. after glucose.....										0.163	0.021				
4 " " "															

\* Calculated as glucose.

bohydrate metabolism of the whole body seems to be least affected by it. It has been shown that with the technique employed in these experiments (50 mg. per kilo of amyntal injected intraperitoneally) in the dog there is no rise in blood sugar, no significant change in the respiratory quotient from the 2nd to the 7th hour after its administration, no effect on the alkali reserve of the blood, and no evidence of adrenal stimulation in the form of chemical regulation of body temperature (Edwards and Page (12), Deuel, Chambers, and Milhorat (13, 14)). The concentration of lactates in the blood is unchanged (unpublished data). While contrary to the results of Long (15) and those of Anderson and Macleod (16), the evidence of Hinsey and Davenport (17) and of Eggleton and Evans (18) distinctly favors the idea that this narcotic causes no serious decrease in muscle glycogen nor increase in muscle lactates. In their recent article on hexosephosphate in muscle (19) Cori and Cori again emphasize the advantages of anesthetizing rather than killing the animal to remove muscle for carbohydrate determinations. Evans, Tsai, and Young (11) found that in cats under amyntal anesthesia for 4 hours the liver may lose about one-half of its glycogen, whereas the loss in the muscle is comparatively small. Large doses, 70 to 160 mg. per kilo, were injected subcutaneously or intramuscularly. Only a slight impairment in carbohydrate utilization was noted by Cori (20) in rats infused with glucose. In the unanesthetized controls 18 per cent of the sugar was deposited as liver glycogen, whereas in the amyntalized animals this fraction amounted to 14 per cent. Hines, Leese, and Barer (21) report that amyntal inhibits in dogs the glycogenesis from continuously injected glucose in the liver but not in muscle.

It is doubtful whether in our experiments the anesthetic seriously interfered with the comparative results on the increase in hepatic glycogen before and after glucose ingestion, as illustrated in Table I by the figures 1.143 and 4.484 per cent. Some indication of the extent of glycolysis in liver and muscle may be gained from the data on soluble carbohydrates and lactates. According to Simpson and Macleod (22) postmortem glycogenolysis yields lactic acid in the muscle, but glucose in the liver; and the normal level of the free sugar in the liver is only slightly below the normal range of blood sugar. On the 22nd day of fast (Table I) soluble carbohydrates in the liver averaged 0.245 per cent or about

0.000 per cent more than the blood (0.032 per cent) or the muscle (0.047 per cent). It is difficult to explain this marked difference between hepatic and blood sugar concentrations as an amytal effect when one considers that the blood sugar level was not elevated, nor have we found the carbohydrate metabolism increased in the dog under these conditions. In the different experi-

TABLE II  
*Carbohydrate Content of Liver and Striated Muscle*

Day of test	Hrs. after glucose	Glucose retained	Muscle					Liver				
			Glycogen*	Soluble carbo-hydrate*	Lactic acid	Total	Difference	Glycogen*	Soluble carbo-hydrate*	Lactic acid	Total	Difference
<i>Dog 36, male, weight 7 kilos; weight of liver 198 gm.</i>												
20		0m.	0.549	0.096	0.057	0.702		0.549	0.484	0.031	1.064	
27			0.442	0.087	0.069	0.608	-0.094	0.827	0.269	0.027	1.123	+0.059
33	6	29	0.554	0.070	0.074	0.698	+0.090	3.132	0.402	0.031	3.565	+2.442
<i>Dog 50, male, weight 5.9 kilos; weight of liver 183.5 gm.</i>												
22			0.325	0.075	0.034	0.434		0.375	0.234	0.011	0.620	
27			0.275	0.067	0.033	0.375	-0.059					
30	4	20	0.331	0.125	0.034	0.489	+0.114	3.326	0.407	0.018	3.751	+3.131
<i>Dog 51, female, weight 6.1 kilos; weight of liver 193 gm.</i>												
20			0.291	0.039	0.018	0.348						
22			0.220	0.047	0.026	0.293	-0.055	1.143	0.245	0.013	1.401	
28	4	25	0.328	0.064	0.040	0.432	+0.139	4.484	0.274	0.020	4.777	+3.376

\* Calculated as glucose.

ments shown in Table II a period varying from 20 to 95 minutes elapsed between the administration of the anesthetic and the excision of the liver samples. There was no apparent correlation between the length of this interval and the amount of soluble sugar in the samples. The low concentration of lactates in the muscle, which was 0.018, 0.026, and 0.040 per cent on the 3 different days

given in Table I, shows that only a small amount of glycolysis took place in this tissue.

### Results

The data on the glycogen, soluble carbohydrate, and lactic acid content of liver and muscle tissues in all the experiments on the three dogs are summarized in Table II. Each figure represents the average obtained from two pieces of the same tissue.

The changes in carbohydrates in the three different dogs are in accord, although the relative values in each animal are different (Table II). In the striated muscle a gradual loss in glycogen with little change in other carbohydrates occurred during fasting. Samples taken 2 days apart from one dog showed a loss in total carbohydrate plus lactate of 0.055 gm. per 100 gm. of tissue. In the other two dogs, when the intervals were 5 and 7 days, the losses were 0.059 and 0.094 gm. per 100 gm. The amounts of glycogen remaining in the muscles after a long fast were 0.442, 0.275, and 0.220 per cent. Other carbohydrates and lactic acid brought the totals to 0.608, 0.375, and 0.293 per cent, respectively. After glucose ingestion the gain in muscle carbohydrates was definite in each case, being respectively 0.090, 0.114, and 0.139 gm. per 100 gm. of tissue. By far the largest part of this increase was in glycogen.

A much greater variation was observed in the liver glycogen during fasting than in that of the muscles. Values of 0.375, 0.549, 0.827, and 1.143 per cent were obtained, bearing no proportionate relationship to the length of fast. Almost as great a variability was found in the sum of the carbohydrates and lactates, which ranged from 0.620 to 1.401 per cent. In Dog 36, in which successive samples of liver were taken, no significant change in total was observed. During the 4 hours after the glucose was ingested a marked increase in hepatic glycogen occurred, amounting to a total of 2.442, 3.131, and 3.376 gm. per 100 gm. of tissue.

Blood was drawn at the time that tissue samples were excised. The concentrations of glucose and lactate in the arterial blood are shown in Table III. During fasting the soluble carbohydrate content of the muscle was quite similar to that of the blood. After the glucose was given the characteristic large increase in blood sugar was seen in each case, whereas there was very little

rise in muscle sugar. Relative figures for blood and muscle in each dog were 152 and 70, 377 and 125, 163 and 64 mg. per cent. Glycolysis probably explains the higher concentration of lactates in the muscle than in the blood, for the liver and blood were not essentially different in lactate concentration.

The cardiac muscles (Table IV) 4 to 5 hours after glucose contained 0.920, 1.407, and 1.808 per cent total carbohydrates.

TABLE III  
*Blood Sugar and Lactates*

Dog No.	Date	Day of fast	Glucose, mg. per 100 cc.				Lactic acid, mg. per 100 cc.			
			Fasting	Hrs. after glucose			Fasting	Hrs. after glucose		
				2	4	6		2	4	
1931										
36	Jan. 26	33	71		192	152*				
50	July 6	27	75				7			
	" 9	30	75	359	377*		11	9	26*	
51	" 22	22	32					7		
	" 27	27	38	-			21			
	" 28	28		372	163*			14	21*	

\* Amytal anesthesia.

TABLE IV  
*Carbohydrate Content of Heart after Glucose Ingestion*

Dog No.	Day of fast	Glycogen*	Soluble carbohydrate*	Lactic acid	Total
		per cent	per cent	per cent	per cent
36	33	0.721	0.069	0.130	0.920
50	30	1.226	0.125	0.056	1.407
51	28	1.689	0.061	0.058	1.808

\* Calculated as glucose.

The glycogen content was considerably higher than that of the striated muscle of the same animal, while soluble carbohydrates were almost identical. Lactic acid was higher in the cardiac muscle, perhaps because autonomic contraction continued after excision.

It was anticipated that the regularly contracting respiratory

muscle might resemble the heart in its carbohydrate content. However, the data of Table I show that the diaphragm is similar to the striated muscle of the leg rather than to the cardiac muscle in this respect. Additional results of the same type were obtained from Dog 50 and from another fasting dog without glucose.

#### DISCUSSION

The most striking and important point which these data demonstrate is that both muscular tissues and liver are able to form glycogen in an apparently normal manner, although the oxidation of sugars during this time is almost completely suppressed. In contrast to the results of Junkersdorf and Mischnat (2) the glycogen increase in the present experiments is closely comparable to that found in rats by Macleod and his collaborators (4) and by Cori (3). The amounts of liver glycogen are similar to those reported by Fisher and Wishart (23), who obtained the

TABLE V  
*Comparison of Results with Those of Other Investigators*

Authors	No. of dogs	Nature of experiment	Glycogen		
			Muscle per cent	Liver per cent	Heart per cent
Schenk (24)	12	Normal diet	0.48-0.57		0.37-0.51
Junkersdorf (25)	4	" "	0.55	6.1	
Fisher and Lackey (26)	3	Meat fed	0.58	1.81	0.50
	2	Fast, 5 days	0.09	0.26	0.28
Junkersdorf (27)	15	" 11 "	0.21	0.59	
Rathery and Kour- ilsky (28)	3	" 6-23 days	0.18-0.51	0.05-3.03	
	2	" 30 days	0.18		0.20
	5	" 30-47 days		0.11-1.83	
Junkersdorf and Mischnat (2)	4	" 11 days	0.21	0.90	0.88
	3	" 26-43 days	0.10	0.40	0.56
This paper	3	" 20-27 "	0.22-0.55	0.38-1.14	

percentages 3.33, 3.30, 3.85, 2.56, and 7.24 in well nourished dogs killed 1, 2, 2, 3, and 4 hours, respectively, after 50 gm. of glucose. The possibility of glycogenolysis in the liver due to the anesthesia has been discussed earlier in the paper. If such occurred at a rate proportional to the amount of glycogen present, or if some glycogen storage from ingested glucose was prevented by the amyta,

there would have been a greater deposit of glycogen than is indicated by our experiments.

It is of interest to compare the amounts of glycogen found in the tissues of the fasted dogs with values reported by other investigators for normal and fasting animals. In addition to the experiments which we have already quoted, a few of the many analyses published for muscle, liver, and heart glycogen are shown in Table V.

The experiments of Schenk (24) are of added interest because he also determined lactic acid and other carbohydrates. He reported from 0.028 to 0.060 per cent lactic acid in muscle and from 0 to 0.067 per cent in heart, while "other carbohydrates" ranged from 0.10 to 0.13 per cent in both. These results are closely comparable to those given in Tables II and IV.

The average muscle glycogen found in our three dogs after 22 to 27 days of fast was 0.312 per cent, slightly higher than most investigators observed after fasts of varying lengths. The liver glycogen values fall within the range of variation found by Rathery and Kourilsky (28) in five dogs fasted between 30 and 47 days (chloralose anesthesia).

Some indication of the fate of the ingested carbohydrate can be obtained from the calculations of the amounts deposited and accounted for in other ways according to figures presented in Table VI. The analysis of the contents of the gastrointestinal tract gives evidence that practically complete absorption of 25 gm. of glucose takes place within 4 hours. The amount of sugar metabolized is estimated on the basis of previous experiments, including those published in 1930 (1) and three other unpublished calorimeter experiments in which the glucose oxidized during the 2nd, 3rd, and 4th hours after glucose ingestion following a 23 to 29 day fast averaged, respectively, 0, 0, and 0.03 gm. per hour.

In calculating the increase in blood sugar it has been assumed that the ratio of the blood volume to the body weight is 0.082 (Chambers and Coryllos (29)), without attempting to allow for a change in volume of blood or tissue fluids after glucose ingestion.

For the total muscle carbohydrate the value of 3:7 given by Palmer (30) as the ratio of the weight of muscle to body weight of normal dogs is used. It is probable for emaciated animals this is more nearly correct than the ratio of 3:10 of Junkersdorf (25).

While it is realized that the carbohydrate content of one leg muscle is not a valid representation of that of the whole striated musculature of the body, on this basis the gain in carbohydrate in the muscle averages a little over 3 gm. If, also, the muscles had continued to lose carbohydrate at the same rate after the second sample was taken (Table II) as they had before, the increase after glucose ingestion would have been from 25 to 100 per cent higher than the amount shown in Table VI.

The increase in liver carbohydrates is based on the sugar concentrations and weights of the organs given in Table II. Approximately 60 per cent of the absorbed glucose is accounted for in Table VI and probably at least 35 per cent is deposited as glycogen in the liver and muscles at a time when practically none of it is being oxidized.

TABLE VI  
*Recovery of Ingested Glucose*

Dog No.	Carbohydrates as glucose									
	Ingested	Vomited	Retained	Present in gastro-intestinal tract	Metabolized (estimated)	Increase in tissues			Excreted in urine	Total accounted for
						Blood	Muscle	Liver		
gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
36	45.6	16.7	28.9	*	0.8	0.46	2.7	4.8	3.8	12.6
50	50.0	29.6	20.4	1.2	0.5	1.46	2.9	5.8	3.1	14.9
51	25.0	0	25.0	0.2	0.5	0.62	3.6	6.5	3.9	15.3

\* Not determined.

In addition to the possibility that more glycogen was stored in the muscles and liver than is calculated, a further fraction of the ingested glucose can undoubtedly be accounted for by storage in other organs and especially in the skin and subcutaneous tissue. Folin, Trimble, and Newman (31) found that following the injection of sugar into dogs the skin contained as much glucose as the blood and more than the muscles. Determinations of glycogen in subcutaneous adipose tissue were made by Hoffmann and Wertheimer (32) and by Seoz (33), who found 2 per cent and even higher in dogs fasted for 2 or more days and then given a diet rich in carbohydrate.

Consideration must be given to the heart as a possible storage

place for fairly large amounts of glycogen. Some results in the literature on heart glycogen of dogs in various states of nutrition were brought together in 1927 by Junkersdorf and Hanisch (34). Individual variations were considerable, but the average values reported by several authors for normal animals ranged from 0.39 to 0.46 per cent, while six dogs which had fasted from 3 to 56 days averaged 0.43 per cent. A more recent article by Visscher and Mulder (35) gives 0.56 per cent as the average for twenty-six normal hearts.

The very high heart glycogen (0.72 to 1.69 per cent) found in our experiments after fasting and one dose of glucose is of the order observed by Fisher and Lackey (26) for depancreatized dogs and by Junkersdorf (36) for phlorhizinized animals. The amounts of total carbohydrate and lactate in the heart (Table IV) are very close to those found in three depancreatized dogs whose tissues were analyzed by the same technique (unpublished data). The hearts from these diabetic dogs contained 1.2 to 1.8 per cent carbohydrate.

#### SUMMARY

Determinations of glycogen, glucose, and lactic acid were made on portions of the liver and muscles of three dogs fasted 3 weeks or more, and on the same tissues 4 to 6 hours after ingestion of glucose.

No serious derangement in the process of glycogen formation was produced by a fast sufficiently long to suppress almost all oxidation of carbohydrate. An increase in total carbohydrate and lactate of 0.1 gm. per 100 gm. of muscle, and of 3 gm. per 100 gm. of liver, occurred after sugar ingestion.

Absorption of 25 gm. of glucose from the alimentary tract was practically complete within 4 hours. The blood sugar reached a maximum in about 2 hours and fell gradually.

Heart glycogen was found to be unusually high, comparable to the values characteristic for pancreatic diabetes, whereas the carbohydrate content of the diaphragm was about the same as that of the striated muscle in the leg.

Of the glucose absorbed about 60 per cent was accounted for and at least 35 per cent was deposited as glycogen in the liver and muscles.

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## **THE EFFECT OF UREA INGESTION ON THE NITROGEN PARTITION OF THE URINE AT ENDOGENOUS NITROGEN LEVEL**

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The possible utilization of urea as a partial substitute for protein in the diet of animals has been the subject of considerable experimentation. The results have, unfortunately, led to a variety of interpretations.

In recent experiments with the ingestion of large amounts of urea by normal human subjects, Moore, Lavietes, Wakeman, and Peters (1) were unable to recover all the urea nitrogen in the excreta. The subjects were first placed in nitrogen equilibrium, in two experiments with fairly high protein diet, in two other experiments in negative balance on low protein diets, and fed urea over a number of days. The ingested urea was not all recovered in the urine. These experiments gave no information concerning the fate of the retained nitrogen. A number of German investigators, Völtz (2), Völtz and associates (3), Honcamp *et al.* (4), and Hanson (5), have claimed retention and utilization of urea in experiments on animals; lambs, sheep, cows. Morgen and his associates (6) were unable to show that ingested urea could replace any part of the protein ration of sheep. Other experiments, carried out on humans, have been concerned with the diuretic effect of urea, or with its use as a measure of kidney function. Most of these experiments were performed on hospital subjects. The experiments of Cathcart and Green (7), as well as those of Addis and Watanabe (8), indicate that by far the greater percentage of urea ingested was recovered in the urine in the first 48 to 72 hours and in all probability would have been completely recovered had the experiments been sufficiently extended. Thus far, the inconclusive results of the experiments

directed to the determination of a possible retention or utilization of ingested urea, may be due to one or another of the following factors. (1) Other forms of nitrogen besides urea were fed daily in the diet in varying amounts so that their value was difficult to determine. (2) The analyses of the excreta were not sufficiently complete to furnish information as to the possible form and amounts of the various nitrogenous end-products. (3) The period of analysis was too short to insure complete recovery of ingested urea. (4) Adequate methods were lacking for the determination of nitrogen loss from the skin. Moreover, in the experiments on ruminants there is some likelihood of bacteria participating in the breakdown of urea in the paunch with the consequent utilization of the nitrogen in some other form.

#### *Plan of Experiments*

The present experiments were planned to determine (1) whether there is any urea nitrogen retention by the normal human subject after ingestion of large amounts of urea when the subject is on a nitrogen-free diet and the nitrogen output is down to the so called minimum or wear and tear level; (2) what effect, if any, such urea ingestion might have on the nitrogen partition of the urine. The advantages of this method of approach to the problem of urea utilization are: (1) The subject at the time of urea ingestion is in a state of nitrogen hunger, a condition most favorable to utilization of any added nitrogen. (2) On such a diet, adequate in calories but practically nitrogen-free, the interpretation of the urinary output becomes of more definite significance. Any added nitrogenous constituent can be quantitatively traced in the urine without the confusing factor of a possible retention of dietary nitrogen or its replacement in the body tissues.

#### *Experimental Procedure*

The urine was collected directly in thoroughly cleaned flasks, in each of which were left 2 cc. of toluene. The flasks and urine with the one exception of the night specimen were kept in the ice box. The night specimen, kept cool, was placed in the ice box immediately on reaching the laboratory. Collections were made from 8 a.m. to 8 a.m. All analyses were made on the same day

the urine was collected with the exception of those for sulfur, which were made at the end of the period. Urines were made up to convenient volume, all rinsings being carefully preserved and mixed with the sample.

Blood was taken for non-protein nitrogen and urea determination for 2 days before urea ingestion, 2 hours after ingestion of urea, and again every 24 hours until the original level was reached.

Feces were collected in three periods, transferred to 1:1 sulfuric acid, and allowed to stand several days. Samples of the resulting uniform fine suspension were taken gravimetrically and total nitrogen determined by the macro-Kjeldahl procedure.

Nitrogen in the diet was determined as follows: The entire ingredients of 1 day's diet, which was identical from day to day throughout the experiments, were carefully weighed and treated with 1:1 sulfuric acid. This gave a thin soup of uniform suspension. Portions were taken gravimetrically for analyses by the macro-Kjeldahl method.

Urine pH was determined daily by the electrometric method with the quinhydrone electrode.

### *Methods*

*Urine*—Total nitrogen was determined by the usual Kjeldahl method. Urea nitrogen was estimated according to Youngburg's modification of the Van Slyke and Cullen method, in which urine is diluted 1:10, ammonia removed by shaking with permutit, urea hydrolyzed by urease solution and aerated into 0.02 N HCl. Ammonia was determined by the Van Slyke and Cullen method; creatinine by the method of Folin; uric acid by the Folin-Wu method; amino acid nitrogen by the Folin colorimetric method; and total sulfur by the Denis modification of the Benedict method.

*Blood*—The non-protein nitrogen was determined by the method of Folin and Wu, and blood urea by Van Slyke and Cullen's modification of Marshall's method.

All analyses were made in duplicate and repeated when adequate checks were not obtained.

Subject R. A. K., age 47 years, male, was a physician in good health. This subject, who was on routine activity, was given a diet containing 3408 calories, which was practically nitrogen-free (0.378 gm. per 24 hours by analysis) except during the first 2 days when the N content was approxi-

mately 3 gm. Details of the diet appear in Table I. When the nitrogen output had reached approximately the endogenous level, namely on the 9th day, 30 gm. of urea were ingested (150 cc. of 20 per cent solution of Baker's c. p., checked by analysis). When the nitrogen output had again fallen to the minimum level, the subject ingested three doses of 50 gm. each of urea, 24 hours apart. The diet was continued until the low level of nitrogen was again reached; a total of 21 days on the protein-free diet.

Observations were made as follows: (1) total daily urinary nitrogen, nitrogen partition of urine (urea nitrogen, ammonia nitrogen, uric acid, creatinine, amino nitrogen, and undetermined nitrogen) and total sulfur; (2) determination of blood non-protein nitrogen and urea throughout the experiment; (3) basal metabolic rates, before and during the course of the experiments; and (4) feces nitrogen in three periods.

The data obtained are given in detail in Table II. The approximate nitrogen minimum was reached by the 9th day of this protein-free diet, namely 2.91 gm. This corresponds with the endogenous nitrogen level reached by the subject of this experiment on a previous occasion (1914), the nitrogen minimum being used as a basis for the determination of the effect of exercise on endogenous protein metabolism (9). However, it is to be expected that this endogenous level would gradually reach a slightly lower level. In the previous experiment referred to, the diet contained 1 gm. of nitrogen daily, whereas in the present experiment it contained only 0.378 gm. While the weight of the subject was approximately the same in both cases, the previous experiments were carried out 17 years ago when the subject was in a state of active athletic training, and this is reflected in the relatively higher creatinine coefficient at that time, namely 8.7, compared with an average of 7.6 in the present experiment. Smith (10) showed in nitrogen minimum experiments that while the approximate nitrogen minimum is reached on a protein-free, high carbohydrate diet in from 5 to 6 days, there is subsequently a gradually decreasing level, of a fraction of 1 gm., extending over a period of 2 weeks. Hence, in arriving at a base-line for our calculation of the amount of ingested urea recovered in the excreta, we have taken the average of the total urinary nitrogen values for the 9th day, 2.91 gm. (the day preceding the ingestion of urea) and the 14th day, 2.18 gm. (the 4th day subsequent to urea ingestion). It would have been ideal to postpone the urea inges-

tion for 4 or 5 days longer to get the actual values, but the difficulties of taking such a diet over a considerable time, and the rationality of estimating the endogenous level from the fore and after

TABLE I  
*Diet Throughout Experiment from 3rd to 21st Days*

Food	Composition	Protein gm.	Carbohy- drate gm.	Nitro- gen* gm.	Fat gm.	Calo- ries
Biscuits (11)	Corn-starch	300	300			1200
	Cane-sugar	25	25			100
	Lard	25			25	225
	Yeast, $\frac{1}{2}$ cake					
Corn-starch pudding	Corn-starch	10	10			40
	Sugar	10	10			40
	Orange juice	20	2			8
Lemonade (2 glasses)	Lemon juice	100	10			40
	Sugar	40	40			160
Sanka	Sugar	24	24			96
	Cream	50	1.5	2	20	194
Jelly		50	37.50			150
Butter		90			77	693
Lettuce		100	1	3		16
Tomato		100	1	3		16
Olive oil		30			30	270
Whisky		15		Alcohol 7.5		60
Sugar		25	25			100
Totals.....		3.5	491.50	0.378	152	3408

\* The nitrogen reading is from an analysis of the total diet.

periods, made this additional delay seem superfluous. The fecal nitrogen, determined during the previous period, as well as during the time of urea ingestion, showed that the urea was completely

## Urea Effect on Nitrogen Partition

TABLE II  
*Effect of Ingestion of Urea on Nitrogen Partition of Normal Subject*  
 R. A. K., height 174 cm.

Date	Body weight	Urine volume	Sp. gr.	pH	Total N	Urea N	Ammonia N	Creatinine N	Uric acid N	Amino acid N	Urea-determined N		Basal metabolic rate	Blood non-protein N	Blood urea mg. per 100 cc.
											gm.	gm.			
Aug.	kg.	cc.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	mg. per 100 cc.	
8	79.5	2054	1.011		13.34	10.85	0.606	0.644		0.054	1.186		-0.5		
9	78.6	2180	1.014		10.00	8.19	0.327	0.587	0.157	0.071	0.668				
10	78.6	1236	1.010		5.37	3.10	0.903	0.574	0.126	0.183	0.484				
11	78.6	1520	1.016		6.29	4.90	0.364	0.566	0.162	0.041	0.257				
12	79.0	1105	1.014		4.56	3.24	0.202	0.542	0.115	0.038	0.423				
13	79.4	1113	1.015		3.43	2.09	0.332	0.579	0.096	0.048	0.285				
14	80.0	550	1.018		5.72	3.06	1.18	0.399	0.607	0.092	0.035	0.747			
15	79.4	1258	1.014		6.40	3.32	1.75	0.397	0.614	0.118	0.046	0.395	1.00	0.354	7.0
16	79.2	1119	1.012		6.93	2.91	1.51	0.333	0.602	0.108	0.061	0.296	1.00	0.335	24.3*
17†	1101	1.024	7.15	10.23	8.78	0.345	0.632	0.119	0.042	0.312	1.00	0.371	-15.75	30.3	10.00
18	79.4	960	1.016	7.28	6.70	5.18	0.343	0.668	0.118	0.041	0.350	1.00	0.378		
19	79.2	1090	1.014	6.82	4.74	3.29	0.376	0.597	0.124	0.055	0.298	1.00	0.368		
20	78.9	1210	1.011	7.10	3.18	1.49	0.375	0.632	0.106	0.064	0.513	1.00	0.350		
21	79.5	343	1.022	6.60	2.18	0.73	0.422	0.598	0.107	0.049	0.274	1.00	0.334		
22§	78.9	1270	1.014	7.35	18.27	17.00	0.429	0.619	0.139	0.054	0.290	0.99	0.312	70.5†	52.7
23§	78.6	1544	1.019	7.28	24.38	23.03	0.417	0.638	0.154	0.054	0.087	0.99	0.293	-17.0	79.8†
24§	78.5	1326	1.023	7.10	24.60	23.23	0.370	0.635	0.138	0.026	0.201	0.99	0.308		35.7
25	79.2	405	1.034	6.62	8.04	6.38	0.421	0.581	0.122	0.040	0.406	0.99	0.310		27.0

26	79.5	486	1.035	6.78	4.19	2.70	0.335	0.547	0.123	0.057	0.428	0.99	0.339	23.4	11.2
27	80.2	586	1.026	6.75	3.41	1.79	0.395	0.581	0.104	0.035	0.505	0.99	0.279	21.8	8.4
28	79.8	1287	1.016	7.30	2.97	1.52	0.391	0.560	0.125	0.089	0.285	0.99	0.306	19.4	7.5
29	79.3	1186	1.013	7.00	2.25	0.92	0.380	0.544	0.111	0.090	0.205	0.99	0.264	18.75	7.3

Urine values represent specimen collected from the previous 24 hours.

\* Immediately before urea ingestion.

† 2 hours after ingestion of urea.

‡ 30 gm. of urea.

§ 50 gm. of urea.

absorbed from the intestinal tract, and hence need not enter further into the calculation of urea recovery. In the first experiment, 30 gm. of urea in 20 per cent solution, containing 14 gm. of nitrogen, were ingested on the morning of August 16, the 9th day of the experiment. The total nitrogen excreted for this day and the subsequent 4 days was 27.02 gm. The average endogenous nitrogen level for this period  $\frac{2.91 - 2.18}{2} = 2.54 \times 5 =$  12.7 gm.  $27.03 - 12.70 = 14.33$  gm. of nitrogen excreted in excess of the endogenous nitrogen. Ingested urea N = 14.0 gm. The calculation for the urea nitrogen excretion is as follows:

	gm.
Urea N on day before urea ingestion.....	1.51
"    "    "    " ending period.....	<u>0.73</u>
	2.24
Daily average endogenous urea N $\frac{2.24}{2} = 1.12$	
Total endogenous urea N for period	
$1.12 \times 5 = 5.60$	
"    urea N put out during experiment. 19.47	
Endogenous urea N.....	<u>5.60</u>
Total ingested urea N recovered.....	13.87
Urea N ingested.....	14.00
Total urea N recovered.....	13.87 = 99.07 per cent

The level of all the other nitrogenous constituents of the urine remained at the same average level during the period of extra urea excretion. The average total sulfur excretion likewise remained constant. This with the trend toward a slightly lower minimum paralleling the lower endogenous nitrogen minimum is further conclusive evidence that in this experiment, not only was the ingested urea fully recovered as such but there was no synthesis of protein or conversion of urea into other nitrogenous forms.

In the second experiment, while the subject continued on the same diet, 50 gm. of urea in 20 per cent solution were ingested on August 21, 22, and 23; a total of 150 gm. of urea, or 70 gm. of nitrogen during the 14th, 15th, and 16th days on the diet. The endogenous base-line was again taken as the average of the day preceding the ingestion of urea and the final low level reached 5 days after the last day of urea ingestion.

In this experiment the total nitrogen output for the 8 days from August 22 to 29 inclusive was 88.11 gm. The total endogenous nitrogen output for the same period  $\frac{2.18 - 2.25}{2} = 2.21 \times 8 = 17.60$  gm.  $88.11 - 17.68 = 70.43$  gm. of total nitrogen excreted during the 8 day period, in excess of total endogenous nitrogen. Ingested urea N = 70.0 gm.

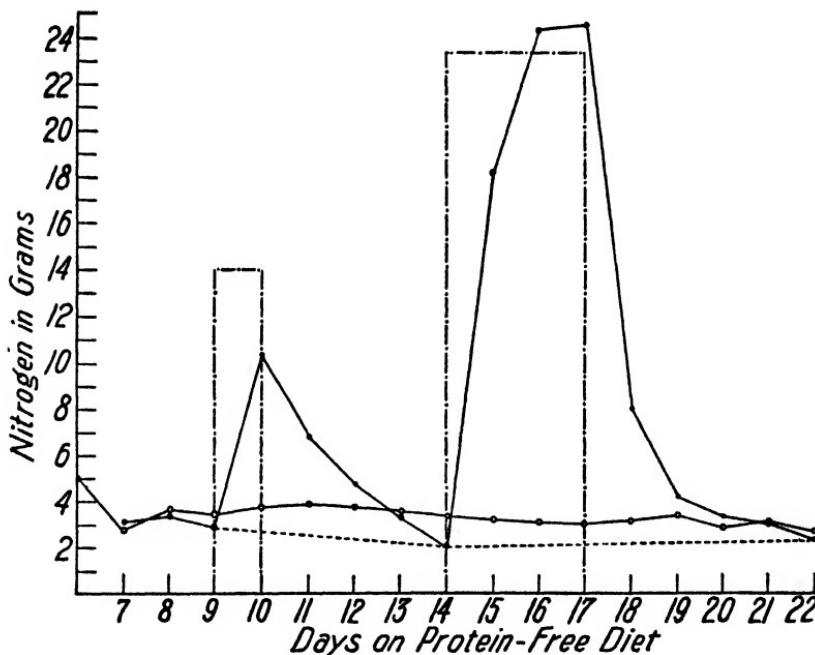


FIG. 1. Curve of total nitrogen and sulfur excretion of a normal subject on nitrogen minimum and after urea ingestion. The solid line indicates total urinary nitrogen; broken line, endogenous nitrogen level; dot and dash line, urea intake; solid line and circle, total urinary sulfur.

The calculations for the urea nitrogen excretion are as follows:

$$\begin{array}{rcl}
 \text{Urea N excreted day before experiment....} & 0.73 \\
 \text{“ “ “ “ ending period.....} & 0.92 \\
 & \hline
 & 1.65 \\
 \text{Daily average endogenous urea N} & 0.82
 \end{array}$$

Total endogenous urea N during experiment	<i>gm.</i>
0.82 × 8 = 6.56	
"   urea N put out during experiment ...	76.57
Endogenous urea N.....	6.56
	<hr/>
Urea N actually recovered.....	70.01
"   " ingested.....	70.0
Total urea N recovered.....	70.01 = 100 per cent

Again, the level of the other nitrogenous constituents of the urine remained practically the same from day to day, likewise the sulfur, thus excluding any possibility of the transformation of urea into other nitrogenous compounds, or its utilization for the synthesis of body protein. The levels of total nitrogen excretion, endogenous nitrogen, total sulfur, and urea ingestion are plotted in Fig. 1.

#### DISCUSSION

It is conclusively shown in these experiments that when urea was fed to a healthy human subject it was quantitatively recovered in the urine as urea. There is a slight delay in the excretion of the ingested urea, which apparently is held in the blood stream and tissues as such. This is evident from the blood urea level, which parallels the delayed output of urea in the urine. It is further apparent in these experiments, from the constancy of the non-urea nitrogen values in the urine partition, as well as in the curve of total sulfur excretion, that ingested urea is not transformed into other nitrogenous constituents, and has no effect on endogenous protein metabolism.

The diet was identical throughout the experiments, the same quantity being taken at each meal each day. Repeated analyses of portions of the food checked the constancy of the daily nitrogen intake. There would not seem to be any advantage in giving urea in larger amounts, or over a longer period of time. In the second experiment, 150 gm. were given in the 3 days and there is no reason to believe, since this amount was entirely excreted as such in the urine, that the body would develop an ability to utilize urea had it been ingested over a longer period. The advantage of using the nitrogen minimum for the study of this problem is quite evident in these experiments. Errors in estimating dietary nitrogen are at once eliminated, as well as possible fluctua-

tions in the nitrogen balance from the variable utilization of dietary nitrogenous constituents from day to day. Abderhalden and others long ago warned against the interpretation of experimental data based on nitrogen equilibrium. The results of these experiments are in accord with those of Addis and Watanabe (8), in humans, and with Morgen and associates (6), Scheunert *et al.* (11), Taylor and Ringer (12), and others on animals.

The discrepancy between our results and those recently reported by Moore, Lavietes, Wakeman, and Peters (1) is more apparent than real. We take the liberty of making the following calculations from the data of their four experiments.

#### *Experiment 1*

The total nitrogen intake for a period of 17 days was 358.2 gm. and the total unrecovered nitrogen, 25.8 gm. = 7.2 per cent of the total intake for 17 days. Of the 358.2 gm. intake, 244.8 gm. represent dietary nitrogen, *estimated as such from standard dietary tables*. The error of this method of estimating nitrogen intake could quite easily be of the magnitude of 7.2 per cent. Of particular interest in this experiment is the fact that during the 10 day period immediately following the 7 days of urea ingestion with an estimated daily dietary intake of 14.4 gm., there was a positive balance of 3 gm., notwithstanding that a delayed excretion of urea nitrogen from the previous period would tend to produce a negative balance. The data in this experiment are not reported day by day, but are lumped for each period, so that it is not apparent at what stage of the after period nitrogen began to be retained. This emphasizes the uncertainty of estimating nitrogen retention on the basis of the nitrogen balance.

#### *Experiment 2*

Daily nitrogen intake of 3.3 gm. for 13 days.....	42.9 gm.
Total urea N.....	243.0 "
	285.9 "

The total unrecovered nitrogen was 7.3 gm. = 2.3 per cent of the total intake.

In this experiment, the nitrogen excretion was determined for only 3 days following the period of urea ingestion. In our own

experiment, with ingestion of 50 gm. of urea daily for 3 days, it is quite evident that at least 1 per cent of the ingested urea may still be excreted on the 4th day following a period of urea ingestion. The remaining 1½ per cent may, in part at least, be due to error in estimation of dietary nitrogen.

### *Experiment 3*

Daily dietary N intake of 3.3 gm. for 13 days.....	42.9 gm.
Total urea N intake, 7 days.....	193.2 "
	236.1 "

The total unrecovered nitrogen was 7.4 gm. = 3.1 per cent of the total N ingested. This is also within the limits of possible error of analysis, plus the fact that dietary nitrogen was estimated from standard food tables.

### *Experiment 4*

The total nitrogen intake for 30 days was 913.2 gm. and the total unrecovered N, 64.5 gm. = 7 per cent of the total intake over a period of 30 days.

388.8 gm. of the total nitrogen intake were estimated from reference to standard tables of food values, which might easily account for a large portion of the 7 per cent of unrecovered nitrogen.

### SUMMARY

Urea as the only source of nitrogen in the diet was fed to a healthy subject in doses of 30 gm. in one experiment and 50 gm. daily for 3 days in a second experiment; the nitrogen excretion at the time of ingestion of urea being at the endogenous level.

The ingested urea was completely recovered as such in the urine; over 90 per cent appearing in the first 24 to 48 hours, the remainder in an additional 24 to 48 hours. The delayed excretion of urea was paralleled by a corresponding increase in blood non-protein nitrogen and blood urea nitrogen. There was no other effect of the ingested urea on the nitrogen partition of the urine nor on the level of total sulfur excretion. The basal metabolic rate decreased on an average of 15.5 per cent on the nitrogen-free, high calorie diet. Ingested urea had no effect on the respiratory exchange.

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## STUDIES ON THE CALCIUM CONTENT OF HUMAN BLOOD CORPUSCLES

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Such early investigators as Schmidt (1) held that human blood corpuscles contain an appreciable amount of calcium. In 1910 Abderhalden (2) concluded from his study of the constituents of the blood of cow, bull, sheep, goat, horse, pig, rabbit, dog, and cat that the corpuscles contain no calcium. The majority of workers have accepted this view and done their work on plasma or serum. However, from 1919 to 1923 Cowie and Calhoun (3) reported 3.47 mg. of calcium per 100 cc. of corpuscles of ox and man; Jones and Nye (4), 8.7 mg. per 100 cc. of corpuscles of normal children; Jones (5) 5.0 mg. per 100 cc. of corpuscles of new born infants; and Cruickshank (6), 1.01 mg. in the corpuscles of dogs per 100 cc. of whole blood. Contemporarily with this group of workers, Richter-Quittner (7) found no calcium in the blood corpuscles of man, cat, ox, goose, horse, dog, or rabbit. In a recent paper Leiboff (8) has confirmed the absence of calcium in human blood cells, and has called attention to the fact that some of the discrepancies noted in the literature may be explained by the unreliability of the methods used.

In view of these conflicting reports, we reinvestigated the problem as related to human blood and studied some of the factors in technique which make for error.

### EXPERIMENTAL

10 to 25 cc. of blood drawn with a dry syringe were collected over sodium citrate.<sup>1</sup> Three or four Van Allen hematocrit tubes

<sup>1</sup> 0.3 cc. portions of saturated sodium citrate were dried in test-tubes over a steam bath. A great excess of this substance appears to interfere with the determination.

were immediately filled and centrifuged at about 2600 R. P. M. for 40 minutes. About two-thirds of the remaining blood was centrifuged at once to obtain the plasma. The whole blood and the plasma calcium were then determined according to one of the schemes noted below.

All measurements of blood, plasma, and reagents were made with volumetric pipettes. Glassware used in the determinations was soaked overnight in dichromate cleaning solution, washed in tap water, distilled water, and alcohol, and allowed to drain in an inverted position until perfectly dry.

Calcium was determined on both the whole blood and the plasma of each specimen. On the assumption that the corpuscles contain no calcium, the theoretical calcium content of whole blood was calculated by multiplying the figure found for the plasma calcium by the percentage of plasma in the sample. If the cells are free from calcium, the experimental and theoretical calcium values for whole blood will check closely; however, if the cells contain calcium, the amount present will be the difference between the experimental and theoretical figures.

Although the study includes some normal individuals, the bloods were taken for the most part from patients in the Colorado Psychopathic Hospital and Out Patient Clinic.

#### *Technique of Calcium Determinations*

Before selecting the methods to be used for calcium determinations, the accuracy of various techniques was extensively investigated. Finally the two procedures outlined below were adopted. We have used more than one method of determination, so that no single error inherent in any one procedure could account for the results found. Throughout our work all determinations were run in duplicate.

*Direct Precipitation from Blood or Plasma*—The plasma calcium was determined essentially according to the method of Kramer and Tisdall (9). To 1 cc. of plasma in a centrifuge tube, 2 cc. of distilled water and 0.5 cc. of saturated ammonium oxalate were added. These were mixed, and the tubes covered to keep out dust and allowed to stand overnight. After centrifugation for  $\frac{1}{2}$  hour at 1800 R. P. M., the supernatant fluid was carefully decanted and allowed to drain from the inverted tube onto filter

paper for at least 5 minutes. (It is essential that the tube be tipped only once and remain in an inverted position after decantation; else the small amount of liquid remaining will tend to stir up some of the precipitate, and this will be lost during the draining. Decanting was found much more satisfactory than siphoning.) The precipitate was washed twice with 5 cc. of ice-cold 2 per cent ammonia water, centrifuged 15 minutes each time, and allowed to drain at least 5 minutes. The mouth of the tube was pressed against the filter paper, and any adhering liquid was carefully wiped away with a soft cloth after each draining. 5 cc. of approximately N sulfuric acid were added to the precipitate, the test-tube was placed in boiling water for 30 seconds (or a little longer if complete solution was not effected), and the contents were titrated with 0.01 N potassium permanganate which had been recrystallized and purified according to the method suggested by Clark (10). The temperature at the beginning of the titration averaged 75°; at the end, 65°. A micro burette accurate to 0.02 cc. was used in the titrations. The end-point was read as the first faint pink color persisting for 30 seconds as judged by comparison against a white background with a similar tube containing water.

Whole blood was determined essentially according to the method of Clark (10). To 5 cc. of blood were added two 5 cc. portions of warm water (65°) with the same pipette used for the blood. After mixing and allowing to stand 20 minutes or longer, 5 cc. each of 1 per cent ammonium chloride and distilled water were added. After thorough mixing and centrifuging for at least 20 minutes in heavy 50 cc. tubes stoppered with paraffined corks, 5 cc. portions of the clear supernatant fluid were precipitated overnight with 1 cc. of saturated ammonium oxalate solution. The remaining procedure was the same as that outlined above for plasma.

*Precipitation from Trichloroacetic Acid Filtrates*—Both plasma and whole blood determinations followed closely those employed by Leiboff (8). 6 cc. of distilled water were added to 3 cc. of blood or plasma; after mixing, the proteins were precipitated with 6 cc. of 20 per cent trichloroacetic acid for 20 minutes or longer. Filtration was effected through Schleicher and Schüll No. 590 filter paper, and 5 cc. portions of the filtrate were precipitated overnight with 0.5 cc. of ammonium oxalate at pH 6.2 (brom-cresol purple).

20 per cent ammonia water was used in neutralizing the filtrate and 0.02 N HCl and ammonia water in adjusting the pH. The remaining procedure was as outlined above for plasma.

Blank determinations run by following through each of the methods outlined on blood or plasma without the addition of

TABLE I  
*Effect of Different Numbers of Washings on Calcium Oxalate Precipitates Isolated from Plasma*

Sample No.	Method of isolation	No. of washings*	Ca recovered	Ca in sample
			from wash water	
1	Direct	1	0.40	10.80
		2	0.60	10.30
		3	1.40	8.37
2	"	1	0.30	15.2
		2	0.42	10.2
		3	1.64	8.6
3	"	1		13.2
		2		9.1
		3		7.8
4	Ashed in platinum	1		8.70
	Direct	2		9.06
5	Ashed in platinum	1		8.95
	Direct	2		8.88
	"	3		7.83

\* 5 cc. of ice-cold 2 per cent ammonia water were used for each washing.

oxalate gave no greater figure than that obtained with the sulfuric acid alone; i.e., 0.02 cc. of permanganate.

*Accuracy of Technique*—After considerable experimentation, two washings of the precipitate of calcium oxalate were employed. Table I shows that, while two washings may result in a slight loss of calcium, the results are more consistent and reliable than when only one is used. With the small amount of wash water recommended for each washing (5 cc.), it is impossible to remove all of

the organic matter in a single washing. Unless removed, this will react with the permanganate solution, and results 25 per cent too high are sometimes obtained. On the other hand, three washings result in a distinct loss, as the calcium determinations on the ashings of our wash water have shown. That the procedure with two washings of the precipitate is satisfactory is shown by the fact that good agreement is obtained between this method and that of ashing in platinum.

Table II shows the necessity of gentle washings with ice-cold ammonia water. Since the amount of calcium contained in each determination averages about 0.1 mg., every precaution must be

TABLE II  
*Effect of Different Methods of Washing Calcium Oxalate Isolated from a Pure Solution of Calcium Chloride\**

Temperature of ammonia wash water °C.	Apparatus used	No. of washings	Calcium	
			mg. per 100 cc.	
20-25	Wash bottle	2	7.62	7.62
			7.62	7.62
20-25	Pipette "	1	9.48	9.44
			2	8.08
20-25	Wash bottle	2	9.34	7.72
			5	9.74
Theoretical.....	Pipette	2	9.74	9.74
				10.00

\* 1 cc. of a standard solution of calcium chloride (1 cc. = 0.1 mg. of Ca) was used for each determination.

taken to prevent loss of the precipitate. It is seen that a rather gentle washing with a pipette is preferable to the direct stream from a wash bottle. With the latter procedure, large amounts of calcium oxalate are sometimes lost, and duplicate determinations tend to give poor checks. Furthermore, the ammonia water used for washing the precipitate must be ice-cold, as the solubility of calcium oxalate, although slight, increases with a rise in temperature. Table II shows also that under the conditions of technique finally selected (two washings with ice-cold ammonia water from a pipette) calcium is recovered almost quantitatively from pure solutions of calcium chloride. Although figures are not reported, good recoveries were also made when calcium was added to blood or plasma.

As a further check on our technique a large amount of blood was drawn from a normal individual. This was divided into three portions, and each was run as a different unknown. Table III shows that the maximum deviation in the average hematocrit readings was 0.39 per cent of cells; in the plasma calcium, 0.23 mg. of calcium; and in the whole blood calcium, 0.05 mg. of calcium.

TABLE III  
*Calcium Determinations on Three Separate Portions of Single Large Sample of Blood Showing Accuracy to Be Expected from Procedures Used*

Portion No.	Blood cell volume <i>per cent</i>	Plasma Ca <i>mg. per 100 cc.</i>	Whole blood Ca <i>mg. per 100 cc.</i>	Calculated whole blood Ca <i>mg. per 100 cc.</i>	Corpuscle Ca* <i>mg. per 100 cc.</i>
40-a	44.00	9.28	5.39		
	44.00	9.34	5.53		
	43.00				
	Average....	43.67	9.31	5.46	5.24
40-b	43.50	9.08†	5.39		
	44.50		5.59		
	44.00				
	44.25				
Average....	44.06	9.08	5.49	5.08	0.41
40-c	44.50	9.14	5.53		
	43.00	9.14	5.49		
	44.00				
	44.00				
Average....	43.88	9.14	5.51	5.13	0.38

\* Figure for calculated whole blood subtracted from average figure of whole blood determinations.

† Duplicate determination lost.

This gives a percentage error of only 0.89 per cent for the hematocrit readings, 2.5 per cent for the plasma calcium, and 0.91 per cent for the whole blood calcium.

#### *Results and Discussion*

In all, twenty-three samples of blood are reported. An inspection of Table IV (Samples 12-a and 12-b, 14-a and 14-b, and 17-a

and 17-b) shows that slightly higher results were obtained by the method of direct precipitation in the presence of blood or plasma

TABLE IV  
*Distribution of Calcium in Plasma and Corpuscles*

Sample No.	Source and sex of donor	Method of isolation	Plasma		Whole blood Ca*	Calculated whole blood Ca	Corpuscle Ca
			Volume per cent	Ca* mg. per 100 cc.			
12-a	Psychopathic inpatient ♂	Direct	54.50	9.45	5.85	5.15	0.70
12-b	" "	CCl <sub>4</sub> COOH	54.50	9.25	5.30	5.04	0.26
13	" "	Direct	50.00	10.72	5.54	5.36	0.18
14-a	" "	"	54.34	9.46	6.20	5.14	1.06
14-b	" "	CCl <sub>4</sub> COOH	54.34	9.35	5.97	5.08	0.89
17-a	" "	Direct	56.50	10.04	6.20	5.67	0.53
17-b	" "	CCl <sub>4</sub> COOH	56.50	9.38	6.16	5.30	0.86
18	" "	Direct	56.75	8.19	5.35	4.65	0.70
19	Normal ♀	"	62.67	9.11	6.44	5.71	0.73
21	Psychopathic inpatient ♂	"	55.63	9.70	5.63	5.40	0.23
22	" "	"	52.38	9.80	5.95	5.13	0.82
23	" "	"	59.63	9.27	6.20	5.51	0.69
24	Normal ♀	"	68.38	9.40	6.80	6.42	0.38
25	Psychopathic outpatient ♂	CCl <sub>4</sub> COOH	65.68	9.55	6.49	6.27	0.22
26	Normal ♀	"	72.63	9.33	7.02	6.77	0.25
27	"	"	66.00	9.32	6.30	6.15	0.15
30	" ♂	"	65.69	9.14	6.11	6.00	0.11
31	Psychopathic outpatient ♂	"	67.75	11.07	8.05	7.50	0.55
32	" "	"	58.10	10.82	7.10	6.29	0.81
33	Psychopathic inpatient ♂	"	52.38	9.91	5.45	5.19	0.26
35	" "	"	54.38	10.09	6.63	5.49	1.14
36	" "	"	53.38	10.91	7.09	5.82	1.27
37	Normal ♂	"	59.25	10.18	6.09	6.03	0.06
38	Psychopathic outpatient ♂	"	60.50	9.91	6.18	5.99	0.19
39	Normal ♀	"	69.88	9.21	7.08	6.64	0.44
40	" ♂	"	56.11	9.20	5.49	5.16	0.33
<b>Average.....</b>							<b>0.53</b>

\* The figures in this column are averages of duplicate determinations.

proteins than after preliminary precipitation of the proteins with trichloroacetic acid. This was confirmed by other determinations not herein reported.

Furthermore, there was a small but consistent difference between the figure for experimentally obtained whole blood calcium and that calculated. This averaged 0.53 mg. per 100 cc. of blood for all determinations: 0.60 mg. for bloods in which the calcium was precipitated directly, and 0.49 mg. for bloods which received the preliminary trichloroacetic acid precipitation. If the amount of calcium calculated had been sometimes greater than the amount found, and sometimes less, the difference might be ascribed in most cases to experimental error; but the consistency of our results, which show more calcium found by direct determination than the amount calculated on the assumption that all of the calcium is in the plasma, does not justify such a conclusion. However, the low magnitude of the figures found for the corpuscle calcium permits us to conclude that, even though the calcium in the corpuscles may have resulted from a corresponding decrease in plasma, any consequent reduction in the plasma calcium figure will be too small to be of clinical significance.

The question arises as to whether the small amount of calcium found in the corpuscles may be explained by the fact that the methods used give slightly low results, as has been mentioned in the early part of this paper. This factor may be disregarded for the following reason. Inasmuch as the same technique was accorded the calcium oxalate precipitates both in the procedure for plasma and in that for whole blood, any errors due to technique in the figures obtained for these determinations would be consistent throughout and would not affect the comparative value of the figures.

#### SUMMARY

1. A study was made of the calcium content of human blood corpuscles in normal individuals and in psychopathic patients.
2. Small amounts of calcium were found in the corpuscles of all bloods examined.
3. The amount of calcium present in the corpuscles is not of enough magnitude to have reduced the plasma calcium values sufficiently to affect their clinical significance.

We gratefully acknowledge the assistance of Dr. Charles A. Rymer in supplying the samples of blood from the Colorado Psychopathic Hospital and Out Patient Clinic.

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# THE PHOSPHORUS CONTENT OF CASEIN

## PRELIMINARY PAPER

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The amount of phosphorus in casein has been a matter of some dispute. Although Bosworth and Van Slyke (2) found that casein, the ash content of which had been lowered by treatment with ammonia and ammonium oxalate, contained only 0.71 per cent of phosphorus, the higher value of 0.80 per cent found by Van Slyke and Baker (9) appears to be more generally accepted. Recently, Carpenter (3) has reported a still higher value of 0.856 per cent phosphorus in the chief constituent of casein prepared by the Van Slyke and Baker method. It seemed desirable to make a further study of this subject. As a first step in this investigation we set out to determine whether the amount of phosphorus in the casein is influenced by the quantity of phosphate in the milk from which it is prepared.

Fresh cow's milk was divided into four portions from which caseins were prepared as follows:

*Casein A*—To 1 volume of milk were added 2 volumes of water and then gradually with constant stirring 0.1 N hydrochloric acid until the isoelectric point was reached.

*Casein B*—Some of the milk was allowed to stand in the refrigerator until it became sour and the casein separated out completely.

*Casein C*—A collodion membrane was filled with milk, a few crystals of thymol were added, and the milk was dialyzed against distilled water for 12 days in the refrigerator. The casein was then prepared from the dialyzed milk by the same method as Casein A.

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*Casein D*—Same as Casein C.

All the caseins after precipitation from the milks were washed with distilled water until the last five wash waters were phosphate-free. The phosphorus contents of the caseins were determined by the Fiske-Subbarow colorimetric method (1). Nitrogen was determined by the Kjeldahl method. The values of the P:N ratios of these caseins are given below.

Casein A.....	0.0522	Casein C.....	0.0444
" B.....	0.0558	" D.....	0.0215 <sup>1</sup>

Caseins A and B which were precipitated in the presence of a large excess of phosphates had higher P:N values than did Caseins C and D, prepared from milks, the phosphate content of which had been reduced by dialysis. That this difference is not due merely to standing is shown by the fact that casein prepared from milk which had been kept in the refrigerator until it soured had a slightly higher P:N value.

We have continued the study of the phosphorus content of caseins prepared from dialyzed milks and have found that it is a relatively simple matter to prepare caseins of much lower phosphorus content than have previously been reported for caseins obtained directly by acidification of milk.

#### *Phosphorus Content of Caseins Prepared from Dialyzed Milks*

Collodion membranes were made by the method described by Simms (7) with the exception that instead of Merck's collodion we used a preparation consisting of 6 gm. of nitrocellulose dissolved in a mixture of 60 cc. of ether and 40 cc. of alcohol. Ten or twelve of these membranes were filled with milk, a few crystals of thymol were added, and the membranes were closed with glass plugs as described by Simms. The membranes were suspended in a flask containing about 2 liters of distilled water saturated with thymol and placed in the refrigerator. For the sake of

<sup>1</sup> Although the milks had been dialyzed for the same length of time, the P:N value of Casein D was less than one-half that of Casein C. Later experiments have also shown that the rate at which the P:N value diminishes is chiefly but not entirely determined by the length of the period of dialysis of the milk. The other factors which enter in are as yet unknown. Rarely, however, are the differences as great as in this case.

convenience this method will be referred to as "plain dialysis." The dialysates were changed each morning. At certain time intervals, two membranes, containing about 80 cc. of milk, were removed; the milk was rinsed into a 250 cc. volumetric flask and diluted to the mark. A portion of this diluted, dialyzed milk, usually 75 cc., was removed, 5 cc. of N sodium hydroxide were added to prevent subsequent curdling, and total nitrogen and phosphorus determinations were made. The rest of this milk was transferred to a beaker and 0.1 N hydrochloric acid added until the pH was 4.6 to brom-cresol green. The casein, which separated out, was washed repeatedly by decantation with distilled water until the last five wash waters showed no trace of

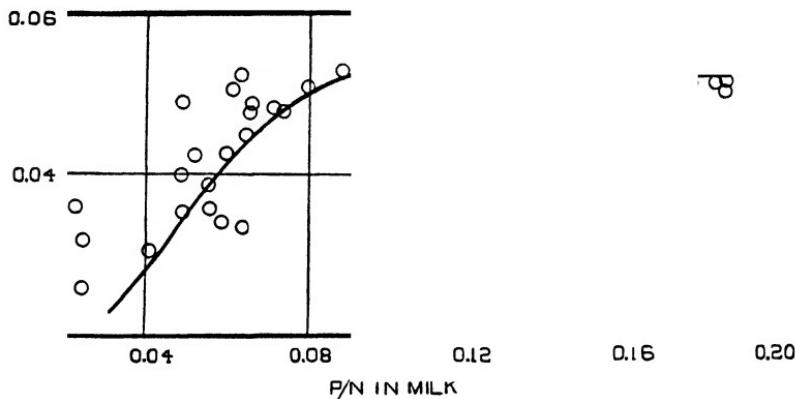


FIG. 1. Relation of P:N in casein to P:N in milk preserved with thymol

phosphate. All the caseins described in the following pages have been prepared by this method. The wet casein was transferred to a 100 cc. volumetric flask, 3 cc. of N sodium hydroxide were added, and the solution was diluted to the mark. Total nitrogen and phosphorus determinations were also made on this solution. The results, which are plotted in Fig. 1, are fairly consistent. This method of dialysis, however, was not satisfactory. The process was very slow, requiring from 2 to 4 weeks to produce a very appreciable lowering of the P:N value of the casein. Moreover, thymol was not an effective preservative, for in most cases the milk soured after 2 or 3 weeks and so could not be used.

The problem was obviously to find a more rapid method of dialysis, as well as an antiseptic which would preserve the milk more effectively and yet not denature the protein. After a survey of the literature, we decided upon the Simms concentrating dialyzer (7) as being best suited to our purpose, since it increased the speed of dialysis without changing the pH of the solution or producing heating effects. Milk containing thymol was dialyzed in the Simms dialyzer. At given time intervals samples were removed and analyzed for total nitrogen and phosphorus. A

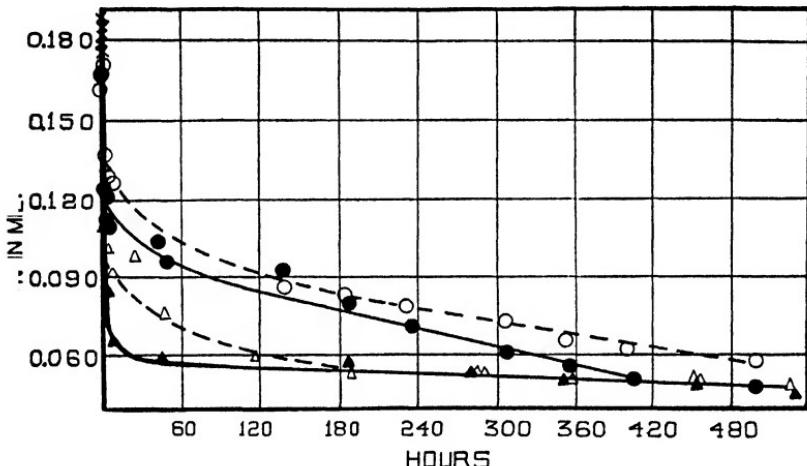


FIG. 2. Change of P:N ratio in milk on dialysis, different methods and preservatives being used. Plain dialysis is represented by the clear symbols, Simms' dialysis is represented by the solid symbols. The cross indicates undialyzed milk; the circle, milk preserved with thymol; the triangle, milk containing 2 per cent NaF.

similar experiment was run with the same milk, plain dialysis being employed. The upper curves in Fig. 2, as well as the data obtained on the 1st day of the dialysis, which are given in Table I, show that in the case of milk the rate of dialysis is increased

\* It was not possible to let the Simms dialyzer run overnight. After about 8 hours dialysis in this apparatus, the membranes were removed, suspended in a flask containing about 2 liters of distilled water, and placed in the refrigerator. The next morning the membranes were again placed in the Simms dialyzer. What is designated here as "Simms' dialysis" is really, therefore, a combination of the two methods.

slightly by using the Simms dialyzer. But even with this apparatus the dialysis still requires considerable time. This slow rate of dialysis is probably due in part to the low solubility of the calcium phosphate. The P:N values of the caseins prepared from these milks agreed with those found previously.

In our next experiments we substituted sodium fluoride for the thymol as an antiseptic. Sodium fluoride is an excellent bactericide. Moreover, the addition of sodium fluoride to the milk should by metathesis result in the formation of calcium fluoride, which is less soluble than calcium phosphate, and the readily diffusible sodium phosphate. That this is very likely what happens is shown by the data in Table I and by the two lower

TABLE I  
*Change of P:N Ratio in Milk on Dialysis, Different Methods and Preservatives Being Used*

Period of dialysis	Thymol		Sodium fluoride (2 per cent)		
	P:N (plain method)	P:N (Simms' method)	Period of dialysis	P:N (plain method)	P:N (Simms' method)
hrs.			hrs.		
0	0.189		0		0.185
1	0.174	0.166	2	0.134	0.109
3	0.138	0.124	4	0.101	0.083
5	0.130	0.113	6	0.090	0.067
7	0.129	0.112			

curves in Fig. 2 which illustrate the change of the P:N ratio on dialysis of milk containing 2 per cent sodium fluoride. Although the rate of dialysis was markedly increased the 1st day, the P:N ratio soon became nearly constant, in contrast to the milk preserved with thymol in which there was a small but definite decrease in the P:N ratio with continued dialysis throughout the same period. However, an unforeseen difficulty was encountered here, for casein prepared from milk which contained 2 per cent sodium fluoride had a higher P:N value, usually about 0.061. Even after a 3 weeks dialysis of such milk, the P:N value of the casein was about 0.055. At the present time we are unable to offer a definite explanation as to the mechanism by which the presence of sodium fluoride in the milk increases the P:N value of the casein.

TABLE II

*Change in Concentration of Total Phosphorus and Total Nitrogen and in the P:N Ratio of Milk on Dialysis, Different Preservatives Being Used*

Milk No.	Period of dialysis	Chloroform			Thymol			Toluene		
		Total milk N	Total milk P	P:N	Total milk N	Total milk P	P:N	Total milk N	Total milk P	P:N
	hrs.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	
32	0				488	92	0.189			
36	0	489	89	0.182	489	89	0.182	489	89	0.182
38	0	512	94	0.184	512	94	0.184	512	94	0.184
40	0	510	89	0.175				510	89	0.175
41	0	506	90	0.178				506	90	0.178
32	1				477	83	0.174			
32	3				463	64	0.138			
32	5				462	60	0.130			
32	7				451	58	0.129			
41	65	447	40.9	0.091				465	44.0	0.095
40	71	461	40.8	0.089				301	27.5	0.091
41	166	440	34.9	0.079				445	37.9	0.085
40	172	437	34.9	0.080				462	38.0	0.082
41	329	383	27.2	0.071				400	30.2	0.076
40	335	394	27.3	0.069				421	30.0	0.071
36	358	380	31.7	0.083	350	24.8	0.071	293	23.4	0.080
38	358	403	30.7	0.076	411	29.1	0.071	312	23.5	0.075
41	496							396	26.2	0.066
40	502							356	23.5	0.066
38	525	382	26.9	0.070	255	16.1	0.063	408	29.1	0.071
36	693	307	21.5	0.070	213	13.3	0.062	261	20.1	0.077
38	694	320	20.7	0.065	187	10.6	0.057	369	23.9	0.065
36	861	288	17.6	0.061	162	9.7	0.060	202	13.7	0.068
38	863	332	19.7	0.059	259	10.8	0.042	388	22.8	0.059
38	1029	279	14.6	0.052	159	6.0	0.038	319	18.0	0.056
36	1080	278	15.9	0.057	127	5.6	0.044	286	18.6	0.065
38	1179	329	16.5	0.050	93	2.9	0.031	348	17.2	0.049
36	1197	257	14.4	0.056	112	4.6	0.041	244	15.0	0.061
36	1365	230	11.6	0.050	106	3.9	0.037	300	15.8	0.053

Because of this effect of sodium fluoride on the P:N value of casein, we abandoned its use for the present and turned our attention again to organic antiseptics. Milk was dialyzed against distilled water by the method of plain dialysis with the preservatives toluene and chloroform as well as thymol in order to compare their effectiveness. Difficulty was experienced in the case of milks preserved with toluene, since this substance attacked the rubber bands around the membranes causing in some cases

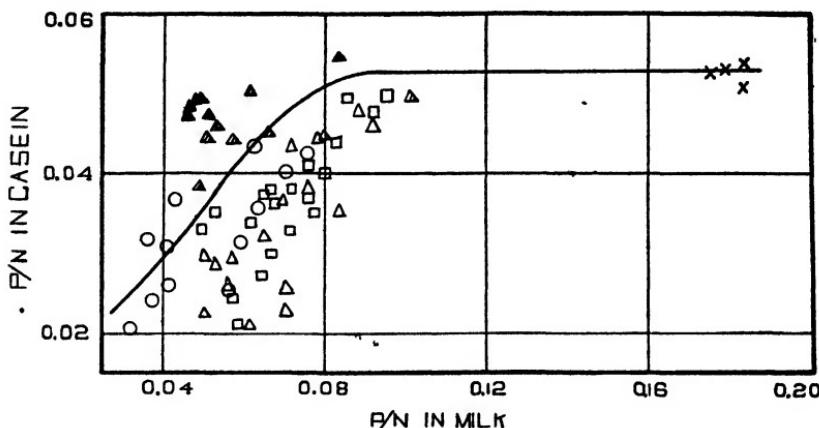


FIG. 3. Relation of P:N in casein to P:N in milk, different preservatives being used. The cross indicates undialyzed milk; the clear triangle, milk preserved with chloroform; the clear square, milk preserved with toluene; the clear circle, milk preserved with thymol; the cross-hatched triangle, milk preserved with chloroform and containing one-third of the total amount of HCl necessary to precipitate the casein; the solid triangle, milk preserved with chloroform and containing two-thirds of the total amount of HCl necessary to precipitate the casein.

considerable loss of milk through leakage. In later experiments metal screw clamps were used to close the membranes, but even with these there was occasionally leakage of milk. The values given in Table II for the concentrations of nitrogen and phosphorus in milks preserved with toluene are, therefore, irregular; in the case of the P:N ratios, however, such losses cancel out and the results are consistent. Chloroform and toluene were superior to thymol in preserving the milk. After 6 weeks, milks containing toluene or chloroform still appeared unchanged, whereas the

milks containing thymol had generally curdled in about one-half that time. As indicated by the data in Table II, milk containing thymol not only lost nitrogen and phosphorus much more rapidly upon dialysis but the values of the P:N ratios were also generally lower than those of the corresponding milks preserved with chloroform or toluene.

The P:N values of caseins prepared from this last series of milks are given by the clear symbols in Fig. 3, in which the P:N ratios of the milks are plotted against the P:N ratios of the corresponding caseins. The curve drawn is the one given in Fig. 1. In general, the points lying farthest to the right of the curve were obtained with milks preserved with toluene or chloroform, while those lying farther to the left were found in the case of milks containing thymol. This shift to the left may have been due to the fact that, since the milks were less effectively preserved when thymol was used, the hydrogen ion concentration of the milks increased, as was shown by the earlier curdling of such milks. The increased acidity in turn caused more calcium phosphate to be dissolved and so removed by dialysis. This increased loss of phosphate obviously lowered the P:N ratio of the milk and so the P:N ratio of the casein prepared from this milk was shifted to the left.

In order to confirm this assumption, we added to fresh milk one-third of the amount of 0.1 N hydrochloric acid necessary for complete precipitation of the casein. This acidified milk was dialyzed, with chloroform as the antiseptic. The loss of total nitrogen on dialysis was the same as that for milk containing chloroform but no added acid. There was a marked increase in the rate of loss of phosphorus at the beginning of the dialysis, however, and hence a lower P:N ratio for the milk. The values for the caseins obtained from this milk are given by the solid triangles in Fig. 3 and are seen to lie, in general, to the left of any caseins previously prepared from milk preserved with chloroform. We repeated this experiment, adding to the milk before dialysis two-thirds of the total amount of 0.1 N hydrochloric acid necessary for complete precipitation of the protein. The loss of phosphorus on dialysis was even more rapid than in the case of milk containing one-half as much hydrochloric acid, while the loss of total nitrogen was the same. The values of the P:N ratios of the

caseins prepared from this milk lie, as one might predict, still farther to the left. It is very probable, therefore, that the differences in the results obtained are due, in part at least, to differences in the acidity of the milk with the consequent differences in the rate of loss of total phosphorus from the milk on dialysis. We are planning a study of the calcium content of such systems, which should give valuable information on this point.

The lowest value obtained for any casein was 0.29 per cent phosphorus, assuming that this casein contained practically the same amount of nitrogen as casein prepared from undialyzed milk, namely, about 15.46 per cent (4). This casein was prepared from milk which had been preserved with chloroform and dialyzed for 36 days before the casein was precipitated. It has not hitherto been possible to separate directly from milk caseins of such low phosphorus content. From the data plotted in Fig. 3 there is nothing to indicate that this low value is the minimum phosphorus content of casein.

Linderstrøm-Lang (5, 6) has recently shown that by repeatedly extracting casein with warm acid-alcohol it is possible to obtain fractions of widely varying composition. Further evidence as to the non-homogeneity of casein has been offered by Svedberg, Carpenter, and Carpenter (8) who have found that casein prepared by the method of Van Slyke and Baker (9) is not a single protein but a mixture of various protein substances of different molecular weights. "Furthermore, samples from separate casein preparations made by this method of preparation, are shown to be different from each other, showing that not only a mixture of proteins is produced by the Van Slyke and Baker method, but a different mixture is to be found in different individual preparations" ((8) p. 709). This being the case, it is possible that the fractions of this mixture of proteins in milk which we designate as "casein" contain different amounts of phosphorus and that they lose phosphorus at different rates on dialysis of the milk. Although our work is still in its initial stages, the results indicate that this phosphorus is much more loosely bound in the casein than has previously been supposed. It is evident, however, that further experimentation will be necessary to determine whether or not this loss of phosphorus from casein on dialysis of the milk is purely chemical in nature or whether it is caused by the action of enzymes.

or of bacteria or is brought about by some combination of these processes.

I wish to take this opportunity to express to Professor Lafayette B. Mendel and Doctor Hubert B. Vickery my sincere thanks for their helpful suggestions and kindly interest in this work.

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# THE APPLICATION OF THE FISKE-SUBBAROW COLORIMETRIC METHOD TO THE DETERMINATION OF PHOSPHORUS IN CASEIN

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In the course of an investigation on the phosphorus content of casein it became necessary to find a method for the determination of phosphorus which would require only small amounts of casein and yet be sufficiently accurate for our work. The Fiske-Subbarow method (2) has been found to be remarkably well suited for this purpose. By this method only 10 or 15 mg. of casein are required for a determination, the values obtained agreeing well with those found from the gravimetric determination of phosphorus. It is much more rapid than the methods commonly employed.

## EXPERIMENTAL

### *Preparation of Casein*

The caseins used in these experiments were prepared from cow's milk by adding slowly with constant stirring 0.1 N hydrochloric acid to a mixture consisting of 2 volumes of milk and 1 volume of distilled water until pH 4.6 was reached. After the casein was allowed to stand for about 1 hour, the supernatant liquid was poured off, and the casein was washed repeatedly by decantation with distilled water until the last five wash waters were phosphate-free. As much of the wash water as possible was then removed by filtration and the casein was washed first with alcohol and then with ether. It was spread out on large watch-glasses and left to dry over a warm radiator for several days. The resulting product was a fine white powder which, from the nitrogen determination, averaged about 90 per cent casein.

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*Determination of Phosphorus in Casein*

*Colorimetric Method*—Approximately 1.5 gm. of casein were transferred to a 100 cc. volumetric flask, 30 cc. of 0.1 N sodium hydroxide were added, and the solution was diluted to the mark. 1 cc. of this casein solution was pipetted into a small platinum dish, evaporated to dryness on the steam bath, and then heated barely to dull redness over a micro burner. A few drops of water and 1 drop of saturated magnesium nitrate solution were added to the residue and this was again evaporated to dryness on the steam bath. The dish was then heated over a micro burner gently at first and then more vigorously until the residue was pure white and brown fumes were no longer given off. In order to remove from the ash any sodium nitrite which might be present, 2 cc. of water were added, warmed gently, and transferred to a 25 cc. volumetric flask. 1 drop of concentrated hydrochloric acid was added to the residue and the dish warmed until solution was complete and the excess acid driven off. Occasionally it was necessary to repeat this treatment with a second drop of acid but usually one drop was sufficient. The dish was then rinsed seven or eight times with 2 cc. portions of warm water, which were added to the first washing. After cooling, the phosphorus content was determined colorimetrically by the method of Fiske and Subbarow. To the 25 cc. volumetric flask were added 2.5 cc. of Molybdate I (2.5 per cent ammonium molybdate in 5 N sulfuric acid) and 1 cc. of the aminonaphtholsulfonic acid reagent. For the standard, 5 cc. of a monopotassium phosphate solution (containing 0.4 mg. of phosphorus) and 65 cc. of water were transferred to a 100 cc. volumetric flask, and 10 cc. of Molybdate I and 4 cc. of the reducing agent were added. The contents of the flasks were diluted to the mark and mixed, and after standing 5 minutes were compared in the colorimeter, the standard being set at 20 mm.

The total nitrogen of the casein solution was determined by the Kjeldahl method. This value multiplied by the factor 6.47, assuming that casein contains 15.46 per cent nitrogen (3), gave the concentration of casein in the solution.

The percentage of phosphorus in the casein is given by the equation:

20

Reading of unknown  $\times$  gm. casein in 100 cc. casein solution = per cent P

The phosphorus contents of nine different casein preparations determined by this method are given in the second column of Table I.

In order to avoid any possibility of the formation of sodium nitrite during the ashing, these determinations were repeated with calcium hydroxide instead of sodium hydroxide to dissolve the casein. To about 0.5 to 1 gm. of casein in a 100 cc. volumetric flask were added 40 cc. of 0.04 N calcium hydroxide and the solu-

TABLE I  
*Phosphorus Content of Casein*

Casein No.	Percentage P in casein		Gravimetric determination
	Colorimetric determination, casein dissolved in NaOH	Ca(OH) <sub>2</sub>	
17	0.79	0.80	0.795
19	0.82	0.82	0.826
20	0.80		0.803
21	0.80	0.78	0.809
22	0.80	0.79	0.820
23	0.80	0.82	0.813
24	0.81		0.793
25	0.78	0.81	0.786
26	0.79	0.79	0.804

tion was diluted to the mark. 2 cc. of this casein solution<sup>1</sup> were ashed in a small platinum dish, the same procedure as above being used. A few drops of water and one of concentrated hydrochloric acid were added to the ash and the solution was evaporated to dryness on the steam bath. The dish was rinsed eight or nine times with 2 cc. portions of warm water which were transferred to a 25 cc. volumetric flask. The rest of the determination was

<sup>1</sup> If 2 cc. of the casein solution are taken for a determination, the equation for the calculation of the phosphorus content of the casein becomes

the same as that in which sodium hydroxide was used to dissolve the casein. The results are given in the third column of Table I.

*Gravimetric Method*—Finally, in order to check the results obtained colorimetrically, the phosphorus contents of the caseins were determined by the gravimetric method as described in the "Report of the Official Agricultural Chemists" (5), the phosphorus being weighed in the form of magnesium pyrophosphate. About 6 gm. of casein were used in each determination. The ashing was carried out in the presence of magnesium nitrate. The results are given in the last column of Table I and agree well with those obtained colorimetrically within the limit of experimental error.

All analyses were made in duplicate and good agreement was obtained both in the colorimetric and in the gravimetric determinations. In the colorimetric determinations there was no blank correction, but in the gravimetric work an average correction of 0.0010 gm. had to be applied to the weight of magnesium pyrophosphate obtained.

An average of these values gives the phosphorus content of casein as 0.80 per cent of the colorimetric method and 0.805 per cent by the gravimetric method. These values are in good agreement with those obtained by Van Slyke and Baker (6), and Bleyer and Seidl (1), and more recently by Linderstrøm-Lang (4).

#### SUMMARY

The Fiske-Subbarow colorimetric method has been applied to the determination of phosphorus in casein and the results are in good agreement with those found gravimetrically. An average of the results obtained with nine different casein preparations gave 0.80 per cent phosphorus by the colorimetric method and 0.805 per cent by the gravimetric method.

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## STUDIES OF THE PEPTIDES OF TRIVALENT AMINO ACIDS

### II. TITRATION CONSTANTS OF TYROSYL-TYROSINE AND OF GLYCYL-TYROSINE

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(Received for publication, December 23, 1931)

#### INTRODUCTION

In studies of the combination of protein with acid and with base attempts have been made on the one hand to correlate the trivalent amino acid composition with the amount of acid or base bound, and on the other hand to correlate certain inflection points on the titration curve with the dissociation capacities of characteristic groups of the constituent amino acids. Investigations such as that of Cohn and Berggren (5) on casein, and of Hitchcock (11) on deaminized gelatin were successful examples of the former type. The relation between the titration constants of proteins and of the free amino acids had been attempted by Cohn (3) before the *Zwitter Ion* nature of the amino acids and proteins had been adequately demonstrated and has since been extensively analyzed by Simms (19) on the basis of the newer concepts of amphotytic dissociation.

That proteins dissociate at ranges definitely removed from those of the corresponding amino acids has now been repeatedly pointed out (4, 9). It therefore seemed preferable to compare the ionizing capacity of the free groups of the protein with that of the free groups of trivalent amino acids, not when the latter are in the free state but when combined in peptide linkage. In a previous communication (9) the titration constants of histidyl-histidine and of aspartyl-aspartic acid were determined. These substances, each containing four free valencies within a rather

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small molecular area reflect, as in the protein, the mutual effect of the crowded free charges upon each other. While no synthetic model may be expected to reproduce in full the properties of the protein molecule, the study of peptides containing the same groups in different spatial relations to each other should render it possible not only to observe dissociation constants comparable to those of proteins, but ultimately to learn something of the distribution of groups on the surface of the larger protein molecule.

In the first study reported the effect was studied of the influence of several carboxyl groups upon each other and upon the amino group and likewise that of imidazole rings upon each other and upon amino and carboxyl groups. Further studies of peptides containing the guanidine nucleus and of peptides with a preponderance of amino groups are now in progress. The present study is concerned with another important free group of the protein, namely the hydroxyphenyl ring of tyrosine, as reflected in the titrations of two peptides of tyrosine, tyrosyl-tyrosine and glycyl-tyrosine.

#### EXPERIMENTAL

*Preparation of Materials*—The tyrosine from which the peptides were synthesized was an Eastman product. Glycyl-tyrosine was prepared according to the method of Fischer (7), and was twice crystallized from alcohol-water mixtures. Nitrogen analysis, according to the method of Koch and McMeekin (14), yielded 11.7 per cent, theory 11.8 per cent.

Tyrosyl-tyrosine was prepared according to the method of Fischer and Schrauth (8). The tyrosine anhydride was crystallized from boiling ammonia and after drying *in vacuo* over  $H_2SO_4$  yielded no amino nitrogen whatever; m.p., 270° uncorrected. Exactly 0.1631 gm. of the anhydride was dissolved in 7.5 cc. of N NaOH and kept for 5 days at about 28°. At the end of this period 7.5 cc. of N HCl were added, and the solution made up to 25 cc. with distilled water. A sample removed for amino nitrogen analysis yielded 4.05 per cent, theory for tyrosyl-tyrosine, 4.07 per cent. The solution was then immediately employed for the titration. Fischer and Schrauth had succeeded in isolating the compound in amorphous form, failing however to give any analytical data. However, on the basis of the previous studies (9) with the anhy-

drides of histidine and of aspartic acid, which showed the complete quantitative conversion of the diketopiperazine to the peptide in the presence of excess alkali, it was decided to treat tyrosine anhydride in a similar manner. Considerably more time and a slightly higher temperature were allowed than in the previous

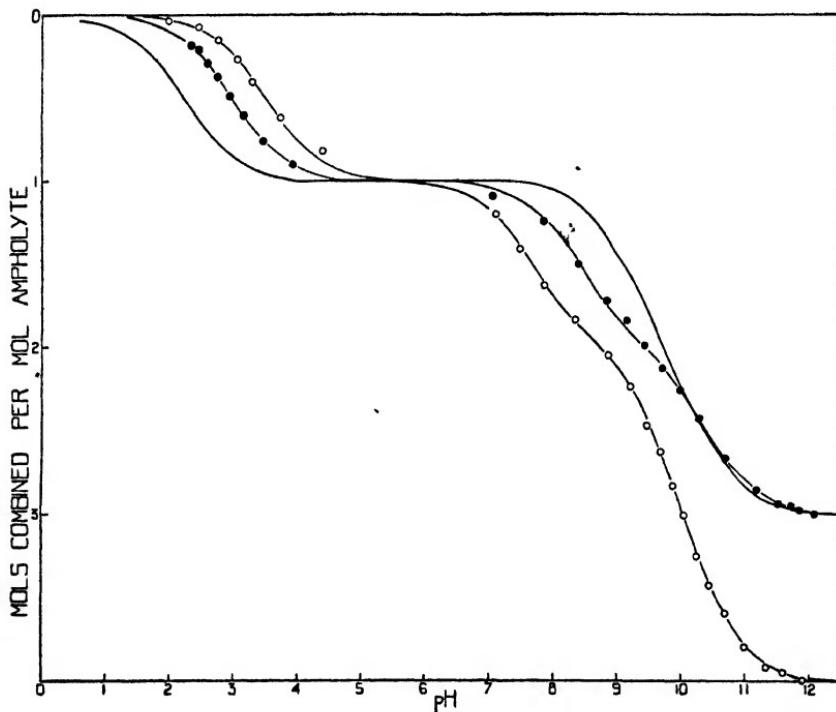


FIG. 1. Curves constructed on the basis of the constants: tyrosine,  $pG_1' = 2.24$ ,  $pG_2' = 9.21$ ,  $pG_3' = 10.28$ ; tyrosyl-tyrosine,  $pG_1' = 3.52$ ,  $pG_2' = 7.68$ ,  $pG_3' = 9.80$ ,  $pG_4' = 10.26$ ; glycyl-tyrosine,  $pG_1' = 2.98$ ,  $pG_2' = 8.40$ ,  $pG_3' = 10.40$ . ○ indicates tyrosyl-tyrosine, ● indicates glycyl-tyrosine.

cases. The satisfactory amino nitrogen analyses together with the titration data, which reveal the stoichiometric combination of exactly four groups, give proof of the structure of this compound. Both peptides, moreover, gave the Millon reaction quite strongly.

*Procedure and Method of Calculation*—For the titration of glycyl-tyrosine, 0.1488 gm. of the compound was weighed and made up

## 468 Peptides of Trivalent Amino Acids. II

to a 0.025 M solution with distilled water. 5 cc. samples were titrated respectively with 0.5 M HCl and 0.5 M NaOH.

Tyrosyl-tyrosine was made up as described in the previous section, yielding a 0.02 M solution in the presence of 0.3 M NaCl.

TABLE I  
*Glycyl-Tyrosine (0.025 M)*

Concen- tra- tion of HCl or NaOH	E.M.F.	pH	pγ	z (experi- mental)	z (calculated)			
					pG <sub>1'</sub> = 2.98 α <sub>1</sub>	pG <sub>2'</sub> = 8.40 α <sub>2</sub>	pG <sub>3'</sub> = 10.40 α <sub>3</sub>	Total Σα = z
<i>N</i>								
0.026	0.4747	2.35	0.058	0.18	0.190			0.19
0.023	0.4814	2.46	0.056	0.21	0.232			0.23
0.020	0.4897	2.60	0.052	0.29	0.294			0.29
0.017	0.4994	2.76	0.049	0.37	0.376			0.38
0.013	0.5101	2.95	0.045	0.49	0.483			0.48
0.010	0.5231	3.17	0.041	0.61	0.608			0.61
0.007	0.5418	3.48	0.035	0.76	0.760			0.76
0.003	0.5693	3.95	0.024	0.90	0.903			0.90
0.003	0.7530	7.06		1.10		0.044		1.04
0.007	0.8003	7.86		1.24		0.224		1.22
0.012	0.8333	8.41		1.50		0.495		1.49
0.017	0.8592	8.85		1.72		0.738	0.027	1.76
0.020	0.8781	9.17		1.84		0.855	0.055	1.91
0.023	0.8944	9.45		1.99		0.919	0.101	2.02
0.026	0.9103	9.72		2.13		0.954	0.173	2.13
0.029	0.9264	9.99		2.26		0.975	0.281	2.26
0.033	0.9442	10.29	0.062	2.43		0.988	0.437	2.42
0.037	0.9685	10.70	0.065	2.67			0.666	2.67
0.042	0.9978	11.20	0.067	2.86			0.863	2.86
0.045	1.0183	11.54	0.069	2.94			0.933	2.93
0.048	1.0300	11.74	0.070	2.95			0.956	2.96
0.051	1.0377	11.87	0.072	2.98			0.968	2.97
0.061	1.0504	12.09	0.077	3.00			0.980	2.98

5 cc. samples were titrated respectively with 0.5 M HCl and 0.5 M NaOH.

The electromotive force measurements and their calculation were the same as described in the previous communication (9). The activity coefficients of H<sup>+</sup> and OH<sup>-</sup> employed for the calculation of the acid and base bound by glycyl-tyrosine were assumed

identical with those with respect to pure HCl and NaOH solutions respectively, and were taken from a smooth curve drawn through the data of Lewis and Randall (16). The activity coefficients for the calculation of acid and base bound by tyrosyl-tyrosine were

TABLE II  
Tyrosyl-Tyrosine (0.02 M)

Concen- tration of HCl or NaOH	E.M.F.	pH	$p\gamma$	z (experi- mental)	z (calculated)				Total $\Sigma\alpha = z$
					$pG_1' =$ 3.52 $\alpha_1$	$pG_2' =$ 7.68 $\alpha_2$	$pG_3' =$ 9.80 $\alpha_3$	$pG_4' =$ 10.26 $\alpha_4$	
<i>N</i>									
0.030	0.4595	1.99	0.06	0.03	0.029				0.03
0.022	0.4820	2.47		0.07	0.082				0.08
0.018	0.4998	2.77		0.15	0.151				0.15
0.015	0.5180	3.08		0.26	0.266				0.27
0.012	0.5315	3.31		0.40	0.382				0.38
0.008	0.5574	3.75		0.62	0.629				0.63
0.0039	0.5967	4.41		0.82	0.885				0.88
0.0039	0.7562	7.11		1.20		0.212			1.21
0.008	0.7785	7.49		1.41		0.393			1.39
0.012	0.8011	7.87		1.63		0.607	0.011		1.62
0.016	0.8297	8.35		1.84		0.824	0.035		1.86
0.020	0.8602	8.87		2.05		0.940	0.105	0.039	2.08
0.024	0.8808	9.22		2.24		0.972	0.208	0.083	2.26
0.028	0.8962	9.48		2.47		0.985	0.324	0.142	2.45
0.031	0.9083	9.68		2.63			0.432	0.208	2.64
0.035	0.9198	9.88		2.84			0.546	0.294	2.84
0.039	0.9308	10.06	0.22	3.01			0.645	0.387	3.03
0.041	0.9415	10.24		3.26			0.734	0.489	3.22
0.044	0.9539	10.45		3.43			0.818	0.608	3.43
0.048	0.9684	10.70		3.60			0.888	0.734	3.62
0.052	0.9861	11.00		3.80			0.941	0.846	3.79
0.056	1.0058	11.33		3.92			0.972	0.922	3.89
0.059	1.0211	11.59		3.95			0.984	0.956	3.94
0.065	1.0392	11.90		4.00				0.978	3.98

experimentally determined in HCl and in NaOH solutions containing the same concentration of sodium chloride as was necessarily present in the solution of the peptide.

In Fig. 1 the ordinate represents the acid and base bound by the peptides, the abscissa the pH. The experimental points are indicated and the curves calculated from the titration constants.

For purposes of comparison, the titration curve of tyrosine, constructed upon the basis of the constants reported by Simms (19) is included with the titration curve of tyrosyl-tyrosine.

The dissociation of each free group is represented by  $\alpha$  in the mass action expression for the behavior of a monovalent acid or base.

$$G' = H \frac{\alpha}{1 - \alpha} \text{ or } pG' = pH + \log \frac{1 - \alpha}{\alpha}$$

where  $G'$  is the titration constant, related to the classical dissociation constant  $K'$  by an expression first clearly presented by Simms (18).

The apparent titration constant  $pG'$  of each isolated group may then be calculated from the pH at which that group is half-neutralized. When, however, two or more groups dissociate simultaneously within the same pH range, then the  $pG'$  values of these groups must be so chosen that at each pH value that the experiments reveal, the sum of the dissociation stages,  $\Sigma\alpha$ , of all such groups will yield the mols of acid or base combined,  $x$ , by the amphotolyte;  $\Sigma\alpha = x$  (Tables I and II).

#### DISCUSSION

The *Zwitter Ion* hypothesis of Bjerrum (1) postulates that the dissociation of carboxyl groups is at acid reactions and of basic groups at alkaline reactions. Tyrosine and its peptides are exceptional in that they possess a group so feebly acid that it dissociates in the extreme alkaline range. This group is the hydroxyphenyl ring which in the case of tyrosine dissociates at a pH slightly greater than 10. The amino group in this amino acid dissociates near pH 9.<sup>1</sup> The carboxyl group, increased in strength by the presence of the cyclic group as in the case of histidine, phenylalanine, and tryptophane appears to have a  $pG'$  value of 2. In the case of glycyl-tyrosine, again two groups

<sup>1</sup> According to Simms, the  $pG'$  value of 10 in tyrosine represents the dissociation of the amino group and that  $pG'$  of 9 the hydroxyphenyl group. The titration studies of Harris (10), however, performed in the presence of formaldehyde, have indicated the acid character of the  $pG'$  10 group. That the substituted benzene ring dissociates at this latter value is brought out in the present communication.

are dissociating at an alkaline reaction and one at acid, whereas tyrosyl-tyrosine contains three groups in the basic range and again one at acid reaction. The titration values and the groups which they presumably represent are given in Table III.

The presence of the peptide bond as shown in the previous communication (9) has the effect of narrowing the range of dissociation of the compound, the resultant of the simultaneous weakening of both acid and basic groups. Thus the very weakly acid hydroxyl group in tyrosine, assigned the  $pG'$  value of about 10,

TABLE III  
Titration Constants  $-25^\circ$

	Carboxyl	Amino	Hydroxyphenyl	Isoelectric point	
Tyrosine (Simms)*.....	$pG_1'$ 2.24	$pG_2'$ 9.21	$pG_3'$ 10.28	pI 5.73	
Tyrosine (Hitchcock).....	$pG_1'$ 2.20	$pG_2'$ 9.11	$pG_3'$ 10.07	pI 5.66	
Glycyl-tyrosine.....	$pG_1'$ 2.98	$pG_2'$ 8.40	$pG_3'$ 10.40	pI 5.69	
Tyrosyl-tyrosine.....	$pG_1'$ 3.52	$pG_2'$ 7.68	$pG_3'$ 9.80	$pG_4'$ 10.26	pI 5.60
Diiodotyrosine (Dalton, Kirk, and Schmidt).....	$pG_1'$ 2.12	$pG_2'$ 7.82	$pG_3'$ 6.48	pI 4.29	

\* Dissociation values of tyrosine were found by Hitchcock (12) to be  $pG_1' = 2.20$ ,  $pG_2' = 9.11$ ,  $pG_3' = 10.07$ . The value of Hitchcock's  $pG_3'$  10.07 is more consistent with the results of this paper than the somewhat higher value found by Simms; namely, 10.28. Kirk and Schmidt (13) give 10.15 as the most probable value of this constant, a result still in harmony with the data in this communication.

has its dissociation somewhat altered in the peptides. This alteration although quite small is nevertheless definite. Glycyl-tyrosine yields a titration value of  $pG_3'$  10.40, characteristic of the weakened aromatic group. Tyrosyl-tyrosine offers a situation analogous to that of histidyl-histidine (9) whereby in the latter compound the feebly basic imidazole groups dissociate at reactions respectively more acid and more alkaline than that of the corresponding group in histidine. If we adopt the  $pG_3'$  value which Hitchcock (12) reported, that of 10.07 for the hydroxyphenyl

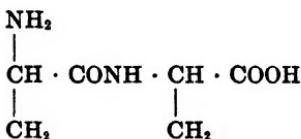
group, rather than that of Simms, then the  $pG'$  values of 9.80 and 10.26 in tyrosyl-tyrosine represent the nearly symmetrical acid and alkaline shifts, respectively more strong and more weak, of the hydroxyphenyl group. The extent of the change in the value of the hydroxyphenyl group, at its greatest only 0.4 in  $pG'$ , must be considered small in comparison with the very much greater alteration in the dissociation undergone by amino and carboxyl groups in the transition from simple acids to peptides (2, 17). In the case of such small peptides as those here considered where no great concentration of amino or carboxyl groups in the neighborhood of the hydroxyphenyl is achieved, no change is made comparable to that noted by Kuhn and Wassermann (15) in the dissociation of the hydroxyl when carboxyl groups are introduced into the benzene nucleus.

The shift of the value for the carboxyl  $pG'_1$  of 2.24 in tyrosine to that of 2.98 in glycyl-tyrosine is characteristic of the influence of the peptide linkage, but the more weakened value for the carboxyl group in tyrosyl-tyrosine, namely  $pG'_1$  3.52, is the resultant not alone of the influence of the acid amide binding but is also due to the introduction into the molecule of the second hydroxyphenyl group. Likewise the more acid shift of the amino  $pG'$  of 9.21 in tyrosine to that of 8.40 in glycyl-tyrosine is consistent with the observed behavior of simple peptides, but the very much weakened value for the amino group in tyrosyl-tyrosine, namely  $pG'$  7.68, is again compounded of the effects of the lengthening of the aliphatic chain and of the introduction of the new cyclic group.<sup>2</sup> The change in value of the amino group from tyro-

<sup>2</sup> The greatly weakened value of 7.68 for the amino group in tyrosyl-tyrosine may be compared with the similarly low value of 7.82 for the basic group in 3:5 diiodotyrosine as obtained by Dalton, Kirk, and Schmidt (6) (Table III). In this interesting halogen derivative of tyrosine, the weakening of the amino group is accompanied by a strengthening of the hydroxyphenyl group, in this case of considerable magnitude; *i.e.*, from 10 in tyrosine to 6.48 in diiodotyrosine. While the effects are not strictly comparable owing to the fact that the halogenation occurs in the benzene nucleus, it is of interest to point out that whereas in both compounds the acid properties of the molecule are increased, in the one by introduction of halogen substituents near the hydroxy group, in the other by introduction of a peptide bond, this effect is in any case at the expense of increasing the strength of the cyclic group and of simultaneously decreasing the strength of the neighboring amino group.

sine to tyrosyl-tyrosine amounts in pG' units to 1.53, which may be compared with the shift of 1.59 units in the change of the amino group in aspartic acid to aspartyl-aspartic acid.<sup>3</sup>

Referring to the structural formula of tyrosyl-tyrosine



it might be assumed that the aromatic group next to the carboxyl would be assigned the pG' value 10.26. This assumption is supported by the pG' value of 10.40 for the hydroxyphenyl group in glycyl-tyrosine in which compound the former group is located near the weakened carboxyl. Further the hydroxyphenyl group next the amino group, would in consequence of the latter's very weakened position, be assigned the stronger more acid value of 9.80.<sup>2</sup> These assignments, while necessarily arbitrary, are con-

<sup>3</sup> Analyzing the effect of the introduction of the second hydroxyphenyl group upon the dissociation value of the amino group, it may be seen that the actual value difference of 9.21 in tyrosine to 7.68 in tyrosyl-tyrosine, namely 1.53 in pG' units, is the resultant of three effects which may be described as follows: firstly, the effect of the peptide linkage on the amino group in the glycyl residue in glycyl-tyrosine which amounts to  $9.21 - 8.40 = 0.81$  units; secondly, the effect of the introduction of the second hydroxyphenyl group on the amino group in glycyl-tyrosine which effect should be analogous to the introduction of the cyclic group in alanine to yield tyrosine which amounts to  $9.72 - 9.21 = 0.51$  units; and thirdly, the modification of the previous step by the alteration in the value of the cyclic group when found in the peptide molecule. This third step may be approximately estimated from the observed shifts of the dissociation values of the aromatic group in tyrosyl-tyrosine from that noted in the amino acid. With Hitchcock's value of pG<sub>s</sub>' 10.07 in tyrosine, the alteration in the value of the hydroxyphenyl groups in the peptide amounts to approximately 0.24 in pG' units. The sum of these effects,  $0.81 + 0.51 + 0.24 = 1.56$ , is comparable with the value of 1.53 found for the shift of the amino group from tyrosine to tyrosyl-tyrosine. The excellent agreement must, of course, be considered as in part fortuitous.

sistent with our notion of the structure of other amino acid complexes.

As in nearly all peptides, the complex is more acid than the constituent amino acids. The isoelectric points of the peptides of tyrosine are, however, but slightly more acid than that of the amino acid itself, the magnitude of such shift being similar to that found in the transition of aspartic acid to aspartyl-aspartic acid.

The hypothesis may be advanced, that whether or not tyrosine be included as a base-binding constituent of proteins depends upon its position in the protein molecule. It appears from this study that tyrosine combined in peptide linkage may be more acid when the hydroxyphenyl group is situated near a number of free amino groups; on the other hand, when the substituted aromatic group is located in the proximity of free carboxyl groups, it may be so very feeble as to combine no base whatever of measurable reactions. This change in strength is illustrated in the statement of Cohn (4), "In gelatin, as in casein, the base combining capacity is slightly higher than the free dicarboxylic acids, but smaller than these plus tyrosine." Further, Cohn and Berggren (5) suggest, "It is conceivable that such amino acids as tyrosine and cystine may retain a certain capacity to combine base when held in polypeptide linkage." It may be that only a fraction of the total number of tyrosine molecules possesses the power of combining with base, depending upon the relative position with respect to the free basic groups within the protein molecule.

It is a pleasure to acknowledge the kind interest and assistance extended me by Professor E. J. Cohn during the progress of these studies in his laboratory.

#### SUMMARY

1. The apparent dissociation constants at 25° have been determined for tyrosyl-tyrosine and glycyl-tyrosine.
2. It is demonstrated that the assignment of the pG' value of about 10 to the feebly acid hydroxyphenyl group in tyrosine is consistent with the behavior of the peptides of this acid.
3. The dissociation of the hydroxyphenyl group depends upon its relative position in the molecule with reference to other free

groups, being increased slightly by proximity to an amino and decreased slightly by the nearness of a carboxyl group.

4. The free amino and carboxyl groups in the tyrosine peptides are weaker as compared with similar groups in the amino acid. The isoelectric points of the complexes are likewise more acid than that of tyrosine itself.

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# RELATIONSHIPS BETWEEN THE STRUCTURE OF ORGANIC COMPOUNDS AND THEIR INHIBITING EFFECT UPON LIVER ESTERASE. RESEMBLANCE TO A LYOTROPIC SERIES OF ANIONS\*

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It has been shown previously (1) that the structure of the hydrocarbon chain, adjacent to a chemically reactive group, plays an important rôle in relation to the inhibiting effect of organic compounds upon liver esterase. The inhibiting effect in many cases appears to be due to the formation of unstable compounds in a reversible system analogous to, and competitive with, that between enzyme and substrate. Changes produced in the surface energy relationships of the dispersed particles would also be expected to alter the activity of an enzyme without involving any direct reaction between an inhibitor and the particular part of the enzyme particle which functions as a catalyst. Such effects should be found to be relatively non-specific for an individual enzyme; *i.e.*, the effect should be general for that type of protein. In studying the effects of simple electrolytes upon enzymes, several investigators, particularly Sherman and coworkers (2) (with pancreatic amylase), have shown a relationship which corresponds to the common lyotropic series of anions. Myrbäck's data indicate such a relationship for salivary amylase (3), and Falk's work (4, 5), with both protease and lipase, indicates a similar relationship. In some cases the effect of individual ions, *e.g.* phosphate, appears to be more specific.

In the present investigation a striking relationship is shown between the inhibiting effects of a series of substitution products of *n*-pentane and the well known effects upon protein dispersion produced by anions corresponding to the substitution radicals.

\* Contribution No. 233 from the Department of Chemistry, University of Pittsburgh.

In other words, the organic compounds have been found to exert an inhibiting effect which is parallel with the common lyotropic series of anions. Such a relationship does not appear to be a coincidence in view of the number of groups studied (including a series of compounds in which the amyl group was replaced by a phenyl group). We believe that this relationship offers an interesting basis for further study of the factors which influence the physicochemical behavior of dispersed proteins, as well as for the specific study of enzyme actions.

A preliminary study has been made of the inhibiting effects of saturated and unsaturated cyclic compounds.

#### EXPERIMENTAL

The general technique of preparing materials and measuring inhibition was the same as that described in a previous paper (1). The velocity of reaction was determined by direct titration at an approximately constant pH of 7.0.

The inhibitor compounds were obtained from the Eastman Kodak Company, with the exception of amyl nitrate. This was prepared from amyl iodide and silver nitrate; a good yield of the product was obtained which distilled at 155°. The other compounds were all tested for purity, and recrystallized or redistilled when necessary.

#### DISCUSSION

From the data in Fig. 1 and Table I, it is evident that, if we consider only the groups attached to the *n*-amyl alkyl radical, the order of inhibiting effect is CN > I > NO<sub>3</sub> > SH > Br > OH > Cl > CO > CONH<sub>2</sub> > NH<sub>2</sub>. We believe that a reasonable interpretation of the group relationship should be in harmony with an explanation of the effects of the corresponding ions upon protein behavior (6). If one accepts the chemical combination point of view in relation to such phenomena, rather than empirical adsorption, the inhibiting effect may be interpreted as a measure of the affinity for the enzyme, since these compounds produce a competitive type of inhibition (7). In view of the correlation between the lyotropic ion series and the dispersion effect upon proteins in aqueous solution, it seems likely that the relationship between the above types of organic compounds and

colloidal enzymes is a similar phenomenon. Since the most effective groups are negative, and those least effective are positive,

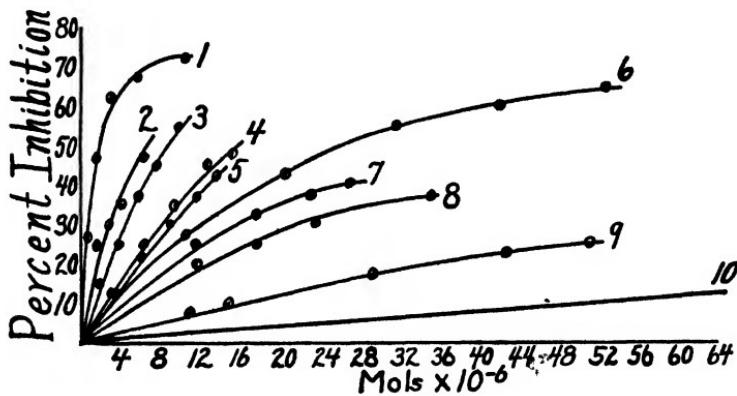


FIG. 1. Inhibitions by amyl compounds. Curve 1, amyl cyanide; Curve 2, amyl iodide; Curve 3, amyl nitrate; Curve 4, amyl mercaptan; Curve 5, amyl bromide; Curve 6, amyl alcohol; Curve 7, amyl chloride; Curve 8, methylbutyl ketone; Curve 9, caproamide; Curve 10, amyramine.

TABLE I  
*Inhibitions of Sheep Liver Esterase by a Series of n-Pentane Substitution Products*

Curve No.	Inhibitor	Mols $\times 10^{-6}$ for 25 per cent inhibition	Inhibition No.*
1	Amyl cyanide	0.6	727.0
2	" iodide	1.3	335.0
3	" nitrate	3.8	115.0
4	" mercaptan	6.1	71.5
5	" bromide	7.4	58.9
6	" alcohol	9.0	48.4
7	" chloride	11.4	38.2
8	Methylbutyl ketone	17.6	24.7
9	Caproamide	50.9	8.6
10	Amylamine	160.0	2.7

\* The ratio of the number of mols of methyl alcohol needed to produce 25 per cent inhibition to the number of mols of another substance needed to produce the same inhibition under the same conditions.

it seems likely that a positive group on the enzyme is the point of attachment.

As shown in Table II and Fig. 2, the order of activity changes only slightly when the same groups are attached to a phenyl radical, the order in this case being I > Br > OH > Cl > NO<sub>2</sub> > CN > CH<sub>3</sub> > CONH<sub>2</sub> > NH<sub>2</sub>. The chief difference noted

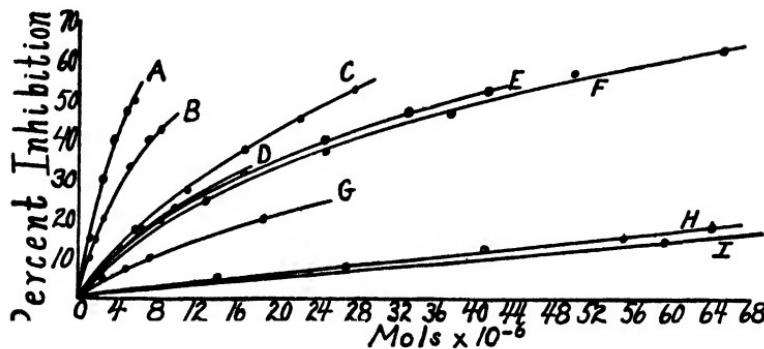


FIG. 2. Inhibitions by phenyl compounds. Curve A, phenyl iodide; Curve B, phenyl bromide; Curve C, phenol; Curve D, phenyl chloride; Curve E, nitrobenzene; Curve F, phenyl cyanide; Curve G, toluene; Curve H, benzamide; Curve I, aniline.

TABLE II  
*Inhibition of Sheep Liver Esterase by a Series of Benzene Substitution Products*

Curve No.	Inhibitor	Mols $\times 10^{-6}$ for 25 per cent inhibition	Inhibition No.
A	Phenyl iodide	2.0	218.0
B	" bromide	3.5	125.0
C	Phenol	9.6	45.4
D	Phenyl chloride	11.4	38.2
E	Nitrobenzene	12.0	36.3
F	Phenyl cyanide	12.5	34.9
G	Toluene	26.0	16.8
H	Benzamide	100.0	4.4
I	Aniline	115.0	3.8

is in the relative position of the CN group. This is probably due to the difference in electronic configuration resulting from the attached phenyl group.

It is interesting to note that although the general order of inhibition found by Falk (5) for anions affecting vegetable lipase

corresponds with that herein cited for organic inhibitors affecting liver esterase, the order of inhibition by methyl and ethyl alcohols

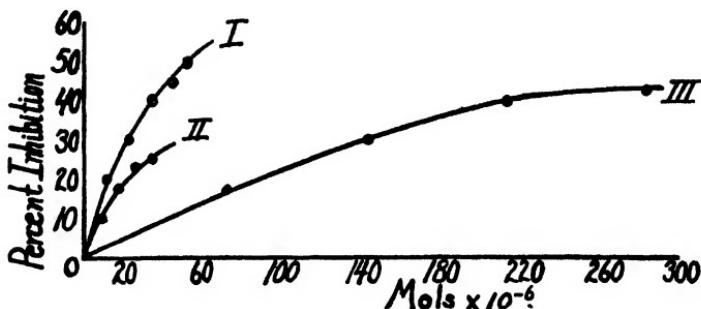


FIG. 3. Inhibition by cyclic compounds. Curve I, cyclohexanone; Curve II, cyclohexanol; Curve III, cyclopentanone.

TABLE III  
*Inhibition of Sheep Liver Esterase by Cyclic Compounds*

Curve No.	Inhibitor	Mols $\times 10^{-6}$ for 25 per cent inhibition	Inhibition No.
I	Cyclohexanone	16.0	27.2
II	Cyclohexanol	34.1	12.8
III	Cyclopentanone	120.0	3.6
	Hexylresorcinol	0.1	3960.0

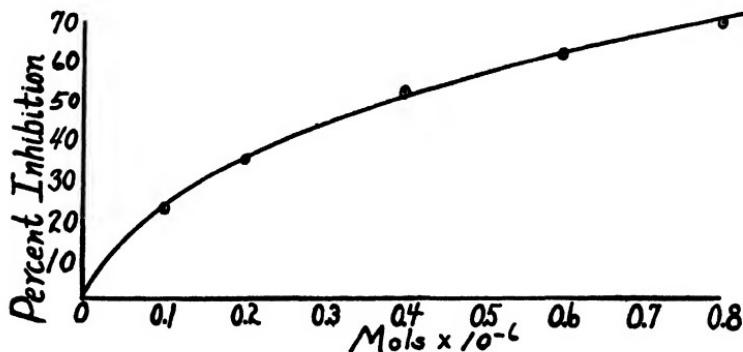


FIG. 4. Inhibition by hexylresorcinol

is reversed (1). The alcohol concentration range was very much lower, however, in the study with liver esterase.

The marked effect of unsaturated groups in the hydrocarbon

ring is shown by the greater activity of phenol compared to cyclohexanol (Table III and Fig. 3). This relationship is further shown by other unpublished work. It will be noted that the analogous cyclic and straight chain compounds, cyclohexanone and methylbutyl ketone, produce very nearly the same effect. The increasing inhibitory power with increasing size of hydrocarbon ring is herein shown by the comparative effects of cyclohexanone and cyclopentanone. This is analogous to the relationship shown previously (1) for straight hydrocarbon chains.

The striking effect of hexylresorcinol (Fig. 4 and Table III) would be expected on the basis of the inhibiting effects of the two negative OH groups, the unsaturated ring, and the aliphatic side chain. In this case, the marked lowering of surface tension is no doubt an important factor which will be considered further in a later paper.

#### SUMMARY

A series of compounds, in which the CN, I, NO<sub>2</sub>, SH, Br, OH, Cl, CO, CONH<sub>2</sub>, and NH<sub>2</sub> groups were attached to an amyl radical, was found to conform in inhibitory effect upon liver esterase to the well known lyotropic ion series for the analogous inorganic ions in their effect upon protein dispersion in aqueous solution. Approximately the same order of activity was found when a phenyl group was substituted for the amyl group.

The unsaturated phenyl radical was found to produce a greater effect than the corresponding saturated cyclohexyl radical.

Increasing the size of the hydrocarbon ring also caused a greater inhibiting effect, as shown by cyclohexanone and cyclopentanone.

Hexylresorcinol produced by far the greatest inhibiting effect of any of the compounds studied.

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## A NEW COLOR TEST FOR CYSTEINE

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### INTRODUCTION

The highly distinctive naphthoquinone test for cysteine, or for cystine after reduction, which has been developed by Sullivan (1), is important for the detection and determination of these sulfur-containing amino acids. Sullivan has found (2) that this reaction requires the presence of three free groups in the molecule, namely —SH, —NH<sub>2</sub>, and —COOH, a fact which partly explains its remarkable specificity.

A similar unusual degree of specificity has now been observed in the reaction of *o*-benzoquinone with cysteine. When an aqueous solution of cysteine hydrochloride is shaken with a chloroform solution of *o*-benzoquinone, a deep red color is produced in the chloroform layer. This coloration is not given by any of the other amino acids and sulfur-containing compounds thus far investigated. Since this test appears to be a specific qualitative reaction for cysteine, and the method of procedure is extremely simple, we are publishing our results for analytical purposes before studying the chemistry of this interesting color reaction in detail.

### EXPERIMENTAL

#### *Preparation of o-Benzoquinone Solution*

The *o*-quinone was prepared by Willstätter's method (3) of oxidizing catechol with silver oxide. A mixture of 0.2 gm. of catechol, 1.5 gm. of anhydrous sodium sulfate, 1.5 gm. of silver oxide (prepared as described by Willstätter), and 10 cc. of cold dry benzene was shaken vigorously for 25 to 30 seconds, and then filtered. The filtrate, when poured into 25 cc. of cold dry petro-

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leum ether, yielded a red precipitate of the quinone. The liquid was decanted from the precipitate, the precipitate washed twice with 2 to 3 cc. of ether, and dissolved in 20 cc. of cold dry chloroform. The resulting solution was pleochromatic, appearing green by reflected light and pink by transmitted light. This chloroform solution of the quinone was stable for 2 or 3 hours if kept stoppered in an ice and salt bath.

To avoid the use of benzene and petroleum ether, which sometimes retained impurities that decomposed the quinone, the reaction was also carried out in ether solution. A mixture of 0.4 gm. of catechol, 1.5 gm. of anhydrous sodium sulfate, 1.5 gm. of silver oxide, and 10 cc. of dry ether was shaken in a test-tube for 35 seconds, then filtered at once. The ether filtrate, chilled in a freezing mixture, deposited red crystals of the quinone. The crystals were separated from the ether solution by decantation, washed once with 1 to 2 cc. of ether, and dissolved in 8 cc. of chloroform. 2 cc. of the freshly prepared solution were used for each test.

#### *Behavior of Cysteine toward o-Quinone*

When 2 cc. of an aqueous solution of cysteine hydrochloride, containing 0.001 gm. of cysteine per cc., were added to 2 cc. of the chloroform solution of the quinone and the mixture shaken vigorously, the pleochromatic color of the chloroform layer was first discharged, giving a pale yellow. On further shaking for about 2 minutes, a deep red color was developed in the chloroform layer. The aqueous layer, colorless at first, gradually became pink on prolonged standing. A blank test made by shaking the chloroform solution of the reagent with water alone produced a colorless chloroform layer and an aqueous layer which slowly turned pink. The aqueous color is therefore due to decomposition of the quinone itself.

The red chloroform layer, when separated from the aqueous portion, dried over sodium sulfate, and filtered from the drying agent, retained the deep color. The color was stable toward 0.1 N hydrochloric acid. The addition of 1 cc. of 0.1 N sodium hydroxide or a few drops of concentrated hydrochloric acid to the colored chloroform layer caused the disappearance of the color in the chloroform and the appearance of a red or purple shade in the

aqueous layer. Therefore, in order to obtain the characteristic color in chloroform, the test was always made in a neutral or weakly acid medium.

The red cysteine-quinone-chloroform mixture is immediately decolorized by reducing agents such as sodium sulfite solution, sodium cyanide solution, or hydrogen sulfide, and is slowly decolorized by shaking with an oxidizing agent such as perhydrol.

*Sensitivity of Color Reaction Produced by Cysteine and o-Quinone in Chloroform*

The limit of dilution of a cysteine hydrochloride solution which will produce a color by shaking with 2 cc. of the chloroform solution of the quinone is shown in Table I.

TABLE I  
*Sensitivity of Cysteine Color Reaction*

Parts of cysteine per million of water	Amount of solution used cc.	Color produced in chloroform layer
1000	2.0	Deep red
100	2.0	" "
100	1.0	Red
10	5.0	Red-brown
10	1.0	Yellow

Table I shows that cysteine cannot be detected by the *o*-quinone test in concentrations smaller than 10 parts per million.

With high concentrations of cysteine, a large excess of the quinone is necessary for the production of the characteristic color. For example, 1 cc. of a cysteine hydrochloride solution containing 0.01 gm. of cysteine per cc. requires 8 cc. of the chloroform solution of the quinone.

*Comparative Reaction of o-Quinone with Cysteine and with Other Sulfur-Containing Compounds*

The tests were carried out by shaking a dilute aqueous solution of each of the sulfur compounds with 2 cc. of the chloroform solution of the quinone. The colors obtained within 10 minutes are

shown in Table II. (On prolonged standing a pink or red color was formed in all the aqueous layers.)

TABLE II  
*Reaction of o-Quinone with Sulfur Compounds*

Compound	Color of chloroform layer	Color of aqueous layer
Cysteine hydrochloride.....	Deep red	Colorless
Cystine      "      .....	Pale yellow	Pink
Glutathione.....	Colorless	"
Ergothionine.....	Pale yellow	"
Cysteic acid hydrochloride.....	Yellow	"
S-Benzylcysteine*.....	"	"
N-Acetyl-S-benzylcysteine hydrochloride*.....	"	"
Diphenylacetyl cystine hydrochloride*.....	"	"
Thioacetic acid.....	Colorless	Colorless
Thioglycollic acid.....	"	"
Thiourea.....	"	"
Ammonium thiocyanate.....	Yellow	Pink
Thiobarbituric acid.....	Colorless	Pale yellow
Thiohydantoin.....	Pale yellow	" "
2-Thiouracil.....	" "	Colorless
4-Methylthiouracil.....	" "	"
p-Nitrophenylmethyl mercaptan.....	Yellow	"
Di-(p-nitrophenylmethyl) sulfide.....	"	"

\* We are indebted to Professor C. P. Sherwin, of Fordham University, for supplying these compounds.

TABLE III  
*Sensitivity of Test with Varying Concentrations of Cysteine and Glutathione*

Parts per million of cysteine	Parts per million of glutathione	Color in chloroform layer
1000	1000	Deep red
500	1000	" "
100	1000	Very faint pink
100	100	Faint pink

The only compound which formed with *o*-quinone a deep red in the *chloroform* layer was cysteine hydrochloride. It is to be noted that the substances which are usually associated with cysteine in

metabolic processes, such as cystine and glutathione, did not give the color in the chloroform layer.

#### *Application of Test to Detection of Cysteine in Presence of Glutathione*

Inasmuch as glutathione reacted with the quinone to give a colorless solution, cysteine could be detected in the presence of glutathione by using a larger quantity of reagent; e.g., 3 to 4 cc. instead of 2 cc. for each test. The sensitivity of the test in determining cysteine in mixtures of glutathione and cysteine is shown in Table III.

It is obvious that the sensitivity of the test is lessened by the presence of relatively large quantities of glutathione.

#### *Reaction of o-Quinone with Amino Acids and Other Nitrogen Compounds Not Containing Sulfur*

The following amino acids were treated with the *o*-quinone reagent according to the same procedure as described for cysteine: serine, glycine, alanine, phenylalanine, glutamic acid hydrochloride, arginine, valine, leucine hydrochloride, lysine hydrochloride, histidine dichloride, tryptophane, and tyrosine. In every case the chloroform layer was colorless or pale yellow, in marked contrast to the deep red formed with cysteine. The aqueous layers became pink or brown.

The following series of other nitrogen compounds was tested similarly with the *o*-quinone reagent: hydantoin, allantoin, iminoallantoin hydrochloride, uracil, aminouracil, barbituric acid, alloxan, alloxanthin, creatine, creatinine, xanthine, and choline hydrochloride. All of these nitrogenous substances produced a pale yellow color in the chloroform. In the case of aminouracil, a deep red was formed in the aqueous, but not in the chloroform layer. The other compounds gave pale yellow, pink, or colorless aqueous layers.

The yellow color of the chloroform part did not interfere with the deep red cysteine color when mixtures of these non-reactive compounds with cysteine were tested for cysteine. In order to determine quantitatively whether the presence of one of these nitrogen compounds would affect the sensitivity of the *o*-quinone reaction for cysteine, tests were carried out on known mixtures of

cysteine and alanine, as shown in Table IV. In each case 2 cc. of the chloroform solution of the quinone were shaken with 2 to 3 cc. of a solution containing the amino acids in the quantities specified below.

The sensitivity of the test in the presence of alanine is therefore the same as in the absence of alanine, given in Table I.

TABLE IV  
*Sensitivity of Cysteine Test in Presence of Alanine*

Parts per million of cysteine	Parts per million of alanine	Color in chloroform layer
100	100	Red
100	1000	"
50	100	"
10	100	Yellow

TABLE V  
*Effect of Various Solvents on the o-Quinone Reaction*

Solvent	Color of cysteine solutions		Color of alanine solutions	
	Solvent layer	Aqueous layer	Solvent layer	Aqueous layer
Chloroform.....	Red, permanent	Colorless	Yellow	Pink
Bromoform.....	" "	"	Orange	Yellow
Carbon bisulfide....	" temporary	"	Colorless	Brown
Benzene.....	Colorless	"	Yellow	Pink
Carbon tetrachloride.	Pink, temporary	Brown	Colorless	Brown

*Effect of Various Solvents on the o-Quinone Reaction*

Samples of *o*-quinone were dissolved in various solvents, and these solutions were each shaken with 1 cc. of a solution of cysteine hydrochloride containing 1 mg. of cysteine per cc. Analogous tests were carried out with the different solutions of the quinone and an equal quantity of alanine.

Although cysteine and the quinone produced a color in the solvent layer in several cases, the color was stable only in the chloroform and bromoform solutions. In the other solutions the color quickly disappeared on continued shaking (Table V).

Alanine and the quinone yielded no deep red in the solvent layers, but an orange color in the bromoform. Since this orange was less easily distinguished from the cysteine color than the yellow alanine color in chloroform, chloroform was shown to be the more suitable medium for the specific cysteine test.

#### *Application of o-Quinone Reaction to Detection of Cystine*

In the absence of cysteine, cystine could be detected by reduction to cysteine and the use of the regular cysteine test.

0.5 cc. of a cystine hydrochloride solution (containing 0.5 mg. of cystine) was added to 1 cc. of concentrated hydrochloric acid and a little mossy tin, and the mixture was heated gently for 2 minutes. The solution was filtered from the tin, diluted to 15 cc., and saturated with hydrogen sulfide. Norit was added, the tin sulfide filtered by suction, and the filtrate boiled to remove hydrogen sulfide. When cooled and neutralized to faint acidity, the solution was shaken with 3 to 4 cc. of the *o*-quinone reagent. A deep red color was produced in the chloroform layer. A blank test on the above reagents gave only a pale yellow chloroform layer.

The limit of sensitivity of this test was found to be 100 parts per million of cystine.

#### SUMMARY

The formation of a red color in the chloroform layer when an aqueous solution of cysteine hydrochloride is shaken with a chloroform solution of *o*-benzoquinone furnishes a simple specific test for cysteine. This reaction may be used to detect cysteine in the presence of cystine, glutathione, and many other sulfur and nitrogen compounds. The test is also applicable to cystine by reduction to cysteine.

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## THE TYROSINE CONTENT OF COCOONS OF VARIOUS SPECIES

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The proteins of silk are distinctive in that they yield on hydrolysis chiefly the monoamino acids, most prominent of which are glycine, alanine, and tyrosine. The high content of tyrosine is particularly striking, values as high as 9 to 11 per cent having been reported (1). For the most part, these analyses of silk have been carried out with samples of Asiatic origin. The method for the determination of tyrosine has usually been a gravimetric one, a method which may be expected to yield lower values than the colorimetric methods recently introduced.

It has seemed of interest to determine the tyrosine content of the cocoons of a number of common species, which are not of commercial importance. The cocoons as obtained were first carefully freed from all visible foreign matter. Total nitrogen was determined by the usual modified Kjeldahl method. The estimations of tyrosine were made in all cases by the use of the modified Millon reaction proposed by Folin and Ciocalteu (2) and modified as a method for small amounts of protein by Folin and Marenzi (3). Moisture and ash determinations were also made. All the values for tyrosine and total nitrogen in Table I are calculated on an ash-free moisture-free basis unless otherwise noted. The results presented are averages of duplicate determinations<sup>1</sup> (in

<sup>1</sup> In view of the fact that individual cocoons were used for each analysis and of the difficulty in completely removing foreign matter (leaves, twigs, etc.) from the cocoon before analysis, the agreement between duplicate determinations was quite surprising. Typical duplicate determinations of tyrosine were cecropia, 11.11 and 11.15 per cent; promethea, 9.43 and 9.49; luna, 8.63 and 8.81; io, 7.35, 7.27, and 7.51.

## Tyrosine Content of Cocoons

a few instances, where available material permitted, of triplicate determinations).

TABLE I

*Tyrosine and Total Nitrogen Contents of Cocoons of Some Common Species*

All values are calculated on a moisture-free, ash-free basis unless otherwise noted.

Family and species	Moisture per cent	Ash per cent	Total N per cent	Tyrosine per cent
<b>Saturniidae*</b>				
<i>Rothschildia orizaba</i> , orizaba silk moth.....	6.42	4.78	16.26	12.01
<i>Samia cecropia</i> , cecropia moth..... “ <i>gloveri</i> , Glover's silk moth.....	6.09	3.61	16.65	12.24
<i>Callosamia promethea</i> , spicebush silk moth.....	6.60	4.51	17.02	12.36
<i>Philosamia cynthia</i> , ailanthus silk moth.....	6.68	2.77	16.60	10.33
<i>Actias luna</i> , luna moth.....	5.69	2.68	16.40	11.38
<i>Telea polyphemus</i> , polyphemus moth.....	6.34	1.16	16.49	9.42
<i>Automeris io</i> , io moth.....	6.33	3.45	16.62	9.27
<b>Bombycidae</b>				
<i>Bombyx mori</i> , silk moth.....	6.99	4.91	16.83	8.38
<b>Lasiocampidae</b>				
<i>Malacosoma americana</i> , American tent caterpillar.....	5.32	2.37	18.24	9.75
<b>Arctiidæ</b>				
<i>Halisidota caryæ</i> , hickory halisidota.....			14.64†	4.55†
<b>Psychidæ</b>				
<i>Thyridopteryx ephemeraeformis</i> , bagworm .....	3.68	4.77	15.13†	4.47†
			16.00	4.42

\* The first five species of this group are classified in the subfamily Attacinae; the last three species in the subfamily Saturniinae.

† Uncorrected for ash and moisture.

It will be noted that the values obtained may be grouped into two divisions. The first group, which includes all the species of the family Saturniidae studied and the silk cocoon of commerce (*Bombyx mori*), had a content of tyrosine similar to that usually

reported for silk fibroin, 9 to 11 per cent. The figures obtained in this investigation are slightly higher, due probably to the difference in results obtained by the gravimetric and colorimetric methods for tyrosine. The second group, which includes the last three species recorded in Table I, showed a much lower content of tyrosine, values of the same order of magnitude as those reported for many common proteins (*e.g.*, serum albumin, edestin, zein, etc.) by Folin and Marenzi (3). It is evident that a high content of tyrosine is not an invariable characteristic of the protein composing the cocoon. It is true that in two of these species of this second group, because of the small size of the cocoon, the available material was not sufficient for determinations of moisture and ash. However, the relatively high values obtained for nitrogen indicate that the values if corrected cannot be of the order of magnitude of those of the first group.

It should be noted that these analyses are for the cocoons as obtained and not for silk fibroin or cocoons treated to remove the silk gelatin (sericin). In the case of *Telea polyphemus*, it was possible to compare the analyses of cocoons after a 2 hour extraction with hot water with the original values. The loss due to removal of water-soluble material (sericin, soluble ash?) was 9.62 per cent. The extracted cocoons contained 9.25 per cent of tyrosine (uncorrected for moisture or ash), a value which if correction had been made would have undoubtedly been higher than the value for the untreated cocoon (9.27 per cent<sup>2</sup>). This was to be expected since silk gelatin (sericin) has been shown to have a content of tyrosine significantly lower than silk fibroin, which comprises the chief protein material of the cocoon.

This investigation forms part of a study, in progress in this laboratory, of the comparative biochemistry of lower forms.

#### SUMMARY

Analysis of cocoons has shown a high content of tyrosine in many of the species studied. A high tyrosine content is not an invariable characteristic of the proteins of the cocoon, since in a number of species, values for tyrosine comparable to those of more common proteins were obtained.

<sup>2</sup> The uncorrected value for tyrosine in this determination was 8.46 per cent.

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## ANSERINE IN MAMMALIAN SKELETAL MUSCLE\*

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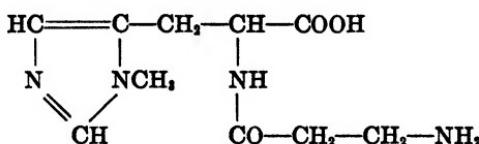
In the course of some experiments on the possible physiological significance of carnosine and related compounds we had occasion to study the content of the former in skeletal muscle from dogs. We expected a large yield of carnosine from this tissue as Krimberg (1) had mentioned a pure carnosine preparation from dog muscle, and as von Fürth and Schwarz (2) had reported "carnosine nitrogen" equivalent to some 4 gm. of free base per kilo of muscle. However, isolation procedures suitable for beef and other tissues yielded less than 35 mg. of base as crude copper salt per kilo of muscle. In one preparation we obtained evidence of the presence of anserine, a methyl carnosine recently discovered in goose muscle by Ackermann, Timpe, and Poller (3) and later isolated from several birds, reptiles (4), and fish (5). These investigators found none in beef (3), and later Keil (6) stated that carnosine but no anserine occurred in mammals, that anserine and possibly carnosine occurred in birds, and that both compounds occurred in reptiles. ("... Bei Säugetieren Carnosin, aber kein Anserin; bei Vogelarten Anserin, vielleicht auch Carnosin; bei Reptilien mitunter Anserin zusammen mit Carnosin (Krokodil), mitunter aber auch nur Carnosin Python und Boa.")

Ackermann and his collaborators have isolated anserine as a copper salt from the lysine fraction of a mercuric sulfate-alcohol

\* A report concerning this work was presented at the Twenty-fifth meeting of the American Society of Biological Chemists at Montreal, April 8-11, 1931 (*J. Biol. Chem.*, **92**, p. lx (1931)).

From the thesis of William A. Wolff presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

precipitate from the aqueous extract of certain muscles (3). The base dried at 120°, analyzed as C<sub>10</sub>H<sub>13</sub>N<sub>4</sub>O<sub>3</sub>, contained one free amino group, one carboxyl group, and one methylimino group (7). Hydrolysis with barium hydroxide yielded *dl*-methylhistidine and β-alanine. Distillation with soda-lime in a stream of hydrogen yielded 1,5-dimethylimidazole identical with Pyman's synthetic product (6-8). Dinitrotolyl anserine on hydrolysis yielded dinitrotolyl-β-alanine (6). These data indicated that anserine is β-alanyl-*N*-methylhistidine, with the imidazole ring substituted in positions (1) and (5) as shown below.



The copper salt of anserine forms a deep blue solution in water and separates out at low temperatures as blue needles, m.p. 230-232°. On drying in air the blue form changes to a second modification, red-lilac in color, which may also be obtained from a hot concentrated alcoholic or aqueous solution. The red form turns blue on drying at 100° *in vacuo* but becomes red again on cooling in air. This characteristic behavior is valuable for identifying anserine.

The free base, usually prepared from the copper salt, crystallizes as colorless needles, is very soluble in water and difficultly soluble in absolute alcohol, melts at 238-239°, reacts as a base, and is dextrorotatory,  $[\alpha]_D^{18} = +11.16^\circ$  to  $11.26^\circ$ . From aqueous solution it is precipitated by phosphotungstic acid, mercuric salts with alcohol, flavianic acid, alcoholic picric acid, and tannic acid. Other derivatives which have been prepared are the nitrate, chloroplatinate, chloroaurate, and monopicrate (3).

Although this new compound has been assumed to be a characteristic extractive of muscles from lower vertebrates (6) we, on the basis of observations mentioned, undertook its preparation from dog muscle. A successful result prompted the examination of muscles from several species of mammals, of which the cat, rabbit, and rat also yielded anserine. No trace of the compound could be obtained in extracts from horse muscle.

*Isolation Procedure*

Preliminary to the study of anserine in mammalian muscle we repeated Ackermann's procedure on chicken muscle. The crude copper salt showed the characteristic properties described above and melted at about 224°.<sup>1</sup> Further purification and the preparation of derivatives was postponed for the time.

The procedure finally adopted for the present study differed only in minor details from that developed by Ackermann, Timpe, and Poller (3). Skeletal muscles dissected from a recently killed animal were ground in a meat chopper and thrice extracted with about 5 volumes of boiling water. The combined extracts were brought to the boiling point, acetic acid was added if necessary, and the protein coagulum removed on muslin. The filtrate was chilled, and after the removal of fat was evaporated over a free flame to about one-twentieth its volume; *i.e.*, to about three-fourths the volume of meat used. The warm viscous solution was slowly poured with stirring into 4 volumes of 95 per cent ethyl alcohol, left in the ice box overnight, and then filtered through coarse paper. The alcoholic filtrate was treated with mercuric sulfate reagent (250 gm. of  $HgSO_4$  and 100 cc. of concentrated  $H_2SO_4$  and water to make 1 liter) as long as a white flocculent precipitate formed. A slight excess of the mercury reagent was indicated by the formation of a yellow granular precipitate, probably a basic mercuric sulfate. The final solution usually contained 60 to 75 per cent alcohol by volume. After standing a few hours most of the alcoholic solution was decanted and the remainder was centrifugalized in 500 cc. bottles. After removing the supernatant fluid the mercury precipitate was thoroughly dispersed by suspending in water and decomposed with hydrogen sulfide—a process sometimes requiring 8 to 10 hours. The solution with washings from the mercuric sulfide was brought to a pH of 4 or 5 with barium hydroxide and filtered.

The filtrate with washings from the barium sulfate was fractionated by the Kossel-Kutscher procedure (9). Silver nitrate was added until a drop of the supernatant liquid removed to a

<sup>1</sup> Melting points reported in this paper are in reality "decomposition ranges." Thermometer readings corrected for stem exposure were checked against a total immersion thermometer certified by the Bureau of Standards.

watch-glass gave a dark brown precipitate with excess barium hydroxide. The precipitate of purine silver formed in the acid solution was removed by centrifugalizing and set aside. The histidine silver fraction, which precipitated on making the solution barely alkaline to litmus with barium hydroxide, was usually removed with the arginine silver fraction which came down on making the solution alkaline to tropeolin O with barium hydroxide. The strongly alkaline filtrate, called the lysine fraction, was neutralized to litmus with sulfuric acid and treated with hydrogen sulfide. The filtrate from the silver sulfide and barium sulfate, usually about 2 liters, was evaporated to 300 cc. on the water bath. About 100 cc. of mercuric sulfate reagent were then added and allowed to stand in the ice box overnight. The precipitate of barium sulfate with some organic material was rejected and the clear solution precipitated with alcohol and additional mercuric sulfate as described above. A second treatment with silver nitrate and barium hydroxide followed by one or two additional mercury-alcohol precipitations was sometimes necessary at this stage, especially if the alkaline solution reduced copper carbonate. Sulfate was removed quantitatively with barium hydroxide from the solution resulting from the decomposition of a final mercury precipitate. Freshly prepared moist copper carbonate added in excess to the final filtrate gave a deep blue solution which was filtered and evaporated on the steam bath. The typical red- or violet-colored copper anserine crystals were filtered on paper in a special centrifugal device.<sup>2</sup>

The copper salt thus prepared was frequently impure, as indicated by the melting point, nitrogen content, and color value (discussed below). For purification the salt was decomposed with hydrogen sulfide, taken through a silver nitrate-barium hydroxide treatment and reprecipitated with mercuric sulfate and alcohol. After decomposing the mercury precipitate with hydrogen sulfide and removing the sulfides and sulfuric acid, the solution was used to prepare the copper salt or was evaporated to 10 to 15 cc. under reduced pressure at 30-35°. For the free base this solution was evaporated further *in vacuo* over sulfuric acid until crystals ap-

<sup>2</sup> This consisted of a Gooch crucible fitted with a rubber collar and short tube permitting it to ride suspended in a centrifuge cup at 1500 R.P.M.

peared; for the nitrate, the solution was neutralized (to litmus) with nitric acid and evaporated until crystals appeared. When crystals of either free base or nitrate appeared 3 volumes of alcohol were added to increase the yield. Losses of anserine were considerable by using this procedure for purification, presumably because the silver-barium treatment precipitates some anserine from concentrated solution.

#### *Color Values*

Our anserine preparations from mammalian skeletal muscle were always contaminated with small amounts of carnosine or substances of similar nature. Since anserine gives no color with the diazo reagent the total chromogenic material present as impurity in any preparation can be estimated by treating a known amount with diazotized sulfanilic acid (10) and matching the resulting color against Hunter's dye standard (11). The amount of color may be calculated as per cent of that which would be produced by an equal weight of carnosine. We have called this value the color value of the preparation. The magnitude of the color value is an excellent index to the degree of contamination by carnosine and similarly reacting substances. A relatively high color value has been found on preparations giving a high nitrogen and low melting point. On treating such a preparation with silver nitrate and barium hydroxide the color value fell and the composition approached the theoretical; also, the color produced with the diazo reagent became more yellow. Although our best preparation gave a slight color with the diazo reagent our experience supports the statement of Ackerman, Timpe, and Poller (3) that repeated treatments with silver nitrate and barium hydroxide will reduce the color value to zero. Our best preparation of free base contained about 0.5 per cent chromogenic material calculated as carnosine. We include this figure in characterizing our preparations because the purity of any sample of anserine or carnosine is open to question unless the color value is reported with other constants.

It should be noted that anserine preparations may also be contaminated with non-chromogenic material as is obviously the case with the preparations from cat and rabbit muscle. Both products gave low color values but the total nitrogen and melting points indicate the presence of considerable impurities. Treatment with

silver nitrate and barium hydroxide did not materially change the nitrogen content of the preparation from rabbit muscle.

### Results

*Dog*—5.40 kilos of skeletal muscle yielded 7.50 gm. of crude copper salt, m.p. 228–230° (corrected). This was recrystallized from water and dried at 120° for analyses.

Total nitrogen (Kjeldahl)

- (a) 0.1100 gm. required 13.93 cc. 0.1 N acid
- (b) 0.1108 " " 13.94 " 0.1 " "

Methylimino group (Herzig and Meyer (12))

0.1106 gm. gave 0.0760 gm. AgI

	Total N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>3</sub> ·CuO.	17.53	4.70
Found.	17.61, 17.73	4.34

*Color Value*—1.5 mg. of salt converted to base and treated with diazotized sulfanilic acid according to Koessler and Hanke's procedure (10) matched 21 mm. of Hunter's (11) dye standard checked against pure histidine.<sup>3</sup> This is 3.5 per cent of the amount of color given by 1.5 mg. of copper carnosine treated similarly. A control experiment with anserine and carnosine in the ratio 100:1 gave the reading calculated for the carnosine plus impurities in the anserine.

*Anserine Nitrate*—Anserine nitrate was prepared from copper salt after purification with silver nitrate and barium hydroxide. Colorless needles separated from 70 per cent alcohol, m.p. 224° (corrected).

Total nitrogen (Kjeldahl modified to include nitrates (13))

- (a) 0.0996 gm. required 16.15 cc. 0.1 N acid
- (b) 0.0994 " " 16.41 " 0.1 " "

Calculated for C<sub>10</sub>H<sub>16</sub>N<sub>4</sub>O<sub>3</sub>·HNO<sub>3</sub>. 23.10

Found. 22.87, 23.08

Color value, 1.7 per cent of theory for carnosine

*Free Base*—Free base was prepared from copper salt after purification with silver nitrate and barium hydroxide. Colorless needles separated from 70 per cent alcohol, m.p. 238° (corrected).

<sup>3</sup> The color standards were prepared and checked by Mr. E. K. Doak.

**Total nitrogen**

- (a) 0.1078 gm. required 17.90 cc. 0.1 N acid  
 (b) 0.1150 " " 19.16 " 0.1 " "

**Amino nitrogen (Van Slyke)**

Barometer reading, 753.6 mm. Hg pressure, at 24.5°

10 mg. gave (a) 1.08 cc., (b) 1.12 cc. in 2 minutes.

**Methylimino group**

0.1054 gm. gave 0.0986 gm. AgI

	Total N	NH <sub>2</sub> -N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>3</sub> .	23.34	5.84	6.24
Found.	23.25, 23.22	5.96, 6.18	5.73

Color value, about 0.5 per cent theory for carnosine

*Cat*—4.20 kilos of skeletal muscle yielded 5.50 gm. of crude copper salt with typical properties, m.p. 224–225° (corrected). Color value, 2.8 per cent. The copper salt was decomposed and treated with silver nitrate and barium hydroxide, and reconverted to copper salt, m.p. 224–225° (corrected).

**Total nitrogen**

- (a) 0.1140 gm. required 14.74 cc. 0.1 N acid  
 (b) 0.1110 " " 14.71 " 0.1 " "

**Methylimino group**

0.1027 gm. gave 0.0696 gm. AgI

	Total N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>3</sub> ·CuO.	17.53	4.70
Found.	18.10, 18.55	4.10

Color value, 0.1 per cent

*Rabbit*—4.40 kilos of skeletal muscle yielded 9.60 gm. of crude copper salt with characteristic properties, m.p. 227–228° (corrected). Color value, 0.9 per cent. The analyses were unsatisfactory. The salt was decomposed and treated with silver nitrate and barium hydroxide. It was then isolated as (a) copper salt, (b) free base.

*Copper Salt*—Copper salt, m.p. 224–226° (corrected), dried at 120° for analyses.

**Total nitrogen**

- (a) 0.1096 gm. required 14.06 cc. 0.1 N acid  
 (b) 0.1250 " " 16.08 " 0.1 " "

**Methylimino group**

0.1398 gm. gave 0.1044 gm. AgI

	Total N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>3</sub> ·CuO.	17.53	4.70
Found.	17.96, 18.01	4.78

Color value, 0.3 per cent

*Free Base*—The free base had a melting point of 222–227° (corrected). Colorless needles separated from 70 per cent alcohol. They were dried at 120° for analyses.

Total nitrogen

(a) 0.1150 gm.	required	19.58 cc.	0.1 N acid
(b) 0.1164 "	"	19.63 "	0.1 " "

Methylimino group

0.1100 gm. gave 0.0979 gm. AgI

	Total N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub> .	23.34	6.25
Found.	23.84, 23.61	5.72

Color value, 0.6 per cent

*White Rat*—75 animals were skinned, eviscerated, the head, feet, and tail cut off, and the entire carcass ground for extraction. The dressed weight was 8.0 kilos, yield 2.0 gm. of crude copper salt with typical properties, m.p. 224–226° (corrected). The salt was dried at 120° for analyses.

Total nitrogen

(a) 0.0916 gm.	required	11.33 cc.	0.1 N acid
(b) 0.0926 "	"	11.52 "	0.1 " "

Methylimino group

0.0851 gm. gave 0.0452 gm. AgI  
0.1404 " " 0.0776 " "

	Total N	(N)-CH <sub>3</sub>
Calculated for C <sub>10</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub> ·CuO.	17.53	4.70
Found.	17.31, 17.42	3.60, 3.53

Color value, 1 per cent

*Horse*—31 kilos of muscle were obtained from the hind limbs. No trace of anserine was found at any stage of preparation. Carnosine was isolated as the copper salt, in characteristic blue hexagons from silver precipitates.\*

#### DISCUSSION

In relation to other nitrogenous extractives, the carnosine content of the skeletal muscles of the horse, ox, and pig (14) is probably second only to creatine. The muscles of these animals contain 0.3 per cent or more of carnosine. The high concentration suggests that it is of importance in muscle metabolism. But, as

\* Mr. W. Earl Graham assisted in this isolation.

colorimetric determinations indicate a much lower concentration in the muscles of many vertebrates (occasionally it appears to be completely absent) Clifford (15) aptly points out that the universal importance of carnosine cannot be postulated.

The discovery of anserine in muscles of birds, reptiles, and fishes by Ackermann and his coworkers focuses attention on a new compound with properties very similar to carnosine. Our demonstration that muscles of certain mammals contain anserine permits the conclusion that neither anserine nor carnosine is associated exclusively with any particular group of vertebrates. The occur-

TABLE I  
*Carnosine and Anserine in Certain Muscles*

Animal	Isolation procedure		Colorimetric determination of carnosine
	Carnosine	Anserine	
	per cent	per cent	per cent
Horse.....	0.18 (16)	None*	0.26-0.48 (14)
Ox.....	0.26 (17)	None (3)	0.32-0.64 (14, 18)
Cat.....		0.09*	0.01-0.49 (19)
Dog.....	Present (1)*	0.10*	
Rabbit.....	0.22 (20)	0.15*	0.03-0.15 (15, 18)
Chicken.....	None (3)	0.12 (3)	0.17 (15)
Boa constrictor.....	Present (4)	None (4)	
Crocodile.....	" (4)	0.03 (4)	
Conger-eel.....	None (5)	0.04 (5)	
River eel.....	0.07 (21)	None (5)	0.37 (15)
Codfish.....	None (5)	0.03 (5)	None (15)

The figures in parentheses refer to the bibliography.

\* This paper.

rence of the two compounds shown in Table I suggests that their functions may be more or less interrelated. In general, muscles high in carnosine are low in anserine and *vice versa*. Accurate comparisons, however, can only be made when more and better data are available. There is some indication that total imidazole content (carnosine plus anserine) may be important for physiological considerations.

#### SUMMARY

Anserine, a methyl carnosine, has been isolated from skeletal muscles of the dog, cat, rabbit, and white rat and identified by

means of the copper salt or nitrate and free base. No trace of the compound could be found in horse muscle. These observations show that anserine is not generally absent from muscles of mammals as had been concluded from a study of ox muscle. The wide-spread distribution of iminazoles, either anserine or carnosine, in vertebrate muscle is discussed.

The authors are indebted to Dr. M. J. Greenman of the Wistar Institute for supplying twenty of the white rats used.

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## THE COMPOSITION OF GALL STONES AND THEIR SOLUBILITY IN DOG BILE

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Gall stones are usually classified into three groups. Of these the solitary cholesterol and pigment stones are rather rare while the mixed or combination stone is by far the most common. Throughout the literature a fairly sharp distinction is made between the solitary cholesterol and the mixed stone, the former consists of almost pure cholesterol while the latter is said to contain varying quantities of other material, notably the calcium salt of bilirubin.

We have made numerous analyses of mixed stones, selecting samples of different color, shape, and size. At times we worked with several hundred gm. that had accumulated in the pathological laboratories and at times with individual samples obtained from gallbladders, at operation. Recently a series of twenty-six such individual specimens were analyzed. Of these seventeen contained 96 per cent or more of cholesterol. The average cholesterol content of all these stones was 94 per cent (Table I). The solitary stone contains between 98 and 99 per cent of cholesterol.

Cholesterol therefore plays the important rôle from a quantitative view-point. Disregarding morphology one might suppose that the inclusion of substances other than cholesterol was purely accidental. As is well known, uric acid, a constituent of kidney stones, though colorless when pure, appears in urine in characteristic brown whet stone formations. Obviously there is an inclusion of urinary pigment.

The residue after extraction of cholesterol consists largely of calcium carbonate and the calcium salts of bilirubin and other

TABLE I  
*Average Composition of "Mixed or Combination" Gall Stones*

	per cent
Cholesterol.....	94
Calcium.....	1.09
Magnesium.....	0.18
Sodium and potassium.....	0.05
Carbonic acid anhydride.....	1.06
Phosphoric acid anhydride.....	0.40
Pigment (by difference).....	3.26
Fatty acids.....	Trace

TABLE II  
*Analytical Data of Gallbladder Bile*

Specimen No.	Solid matter	Cholesterol	Cholesterol Solid matter	Cholesterol after saturation	Increase of cholesterol
<b>Human bile</b>					
608	per cent	mg. per cent	per cent	mg. per cent	mg. per cent
612	4.95	112	2.26	133	21
613	15.0	364	2.42	408	44
614*	18.0	610	3.38	800	190
614*	4.75	86	1.85	103	17
102	4.4	90	2.05	110	20
103	12.9	125	0.97	160	35
104	10.7	330	3.08	370	40
105	13.5	300	2.22	350	50
106	1.85	50	2.70	65	15
<b>Average</b> .....		229		278	48
<b>Dog bile</b>					
63	24.79	70	0.28	180	110
64	25.02	130	0.52	320	190
56	22.0	8	0.03	134	126
57	15.0	8	0.05	96	88
58	22.8	2	0.01	288	286
59	16.7	2	0.01	428	426
72†	21.28	2	0.01	415	413
73	18.92	20	0.10	70	50
75	23.8	82	0.34	208	126
84	16.47	44	0.27	138	94
<b>Average</b> .....		36.8		228	190.9

\* Mixture of thirty bile specimens obtained at autopsy.

† Mixture of two specimens.

bile pigments. No bile salts and only traces of fatty acids were found.

### *Solubility of Gall Stones in Dog Bile*

The interesting observation that human gall stones dissolve in the gallbladder bile of the dog was first described by Naunyn (1) and amply confirmed by Harley and Barratt (2), Hansemann (3), and most recently by Walsh and Ivy (4). The reason for this phenomenon has never been determined.

In the experiments embodied in Table II, the cholesterol content of the gallbladder bile of the human and dog was determined by the gravimetric method of Windaus before and after saturation with cholesterol. Saturation was accomplished by prolonged shaking with the finely powdered substance.

It is seen that while cholesterol of dog bile is low, both absolutely and in proportion to solid matter, yet this bile dissolves 4 times as much cholesterol as human bile. This we believe is the chief cause of the solution of human stones in the gallbladder bile of the dog. In confirmation of this theory, it was found that stones were insoluble in dog bile that had been saturated with cholesterol.

### SUMMARY

1. The average cholesterol content of a large series of the common mixed or combination gall stones was 94 per cent.
2. The solubility of human gall stones in dog bile is due to the low initial cholesterol content of the dog bile. This is the reason for its relatively high solubility coefficient for this substance.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### I. THE DETERMINATION, BY SIMPLE ABSORPTION, OF CARBON DIOXIDE, OXYGEN, AND NITROGEN IN MIXTURES OF THESE GASES

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### APPLICABILITY OF MANOMETRIC APPARATUS TO ANALYSIS OF GAS MIXTURES

In the original description of the manometric blood gas apparatus by Van Slyke and Neill (1924) it was pointed out that the apparatus could be used for air analyses. Details for adaptation of the apparatus to the different gas mixtures encountered in biological analyses were not, however, perfected at that time. Such details are now available.

The constituents of a gas mixture can be successively removed by absorption or combustion, and the amounts so removed estimated by the decrease in gas pressure registered on the manometer.

The absorption, in the cases of  $\text{CO}_2$  and  $\text{O}_2$ , can be carried out by admitting the necessary solutions to the chamber itself, so that transfer of the gas to other vessels for absorption is avoided. Such transfer can, however, readily be accomplished to modified Hempel pipettes when desirable.

Another procedure, especially adapted to estimation of a gas forming only a minute proportion of a mixture, is based on isolation of such a gas by absorbing it in the chamber of the apparatus with suitable reagents, such as alkali for  $\text{CO}_2$ , or reduced blood for  $\text{CO}$ . The unabsorbed gases are then ejected, and the absorbed gas is set free from solution and measured, as in blood gas determinations.

Analysis by combustion is carried out as easily as with ordinary volumetric apparatus. The manometric apparatus has an ad-

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vantage in analysis of highly explosive mixtures, which in other methods are ordinarily diluted with inert gas to reduce their explosibility. Here such dilution and its resultant loss of accuracy are unnecessary, because the explosibility can be decreased merely by putting the mixture under reduced pressure during ignition.

In convenience, analysis of gas mixtures by the manometric apparatus is not inferior to methods of equal accuracy by the usual procedures with gas burettes and absorption bulbs. When the absorbing solution can be admitted into the chamber of the manometric apparatus, making gas transfer unnecessary, the manometric procedure is likely to be the more convenient.

In the maximum accuracy attainable, when simple absorption procedures are used to remove the gases determined, the manometric technique is inferior to the more refined types of volumetric methods. A 0.1 mm. division on the 500 mm. length of manometer ordinarily employed corresponds to 0.0002 of the gas measured, and marks the limit of manometric accuracy in this type of analysis. Volumetric gas burettes, on the other hand, for special purposes can be made with relatively large volumes and with small bores at the points where measurements are made, so that a 0.1 mm. division corresponds to 0.0001 of the gas measured, as in the ordinary 10 cc. Haldane apparatus, or to a still smaller fraction if it is desired. For many purposes, however, the accuracy of 0.0002 suffices, and the convenience of the manometric procedure then may make it the one of choice.

Furthermore, the manometric technique is not limited to simple absorption procedures. The isolation method with the manometric apparatus makes possible a great extension of its refinement, and permits determination, without unusual precautions, of minute proportions of gases, with an accuracy such as can be attained with volumetric methods only by extreme precautions and use of special apparatus. Thus CO<sub>2</sub> in atmospheric air can easily be determined manometrically by the isolation method with a precision approaching 0.0003 of a volume per cent of the air, and small amounts of carbon monoxide to 0.001 volume per cent.

The manometric method also has an advantage for determining small amounts of inert gas admixed with great amounts of other gases capable of removal by absorption. In such a case the sample can be measured by its pressure at 50 cc. volume, and the

residual unabsorbed gas by its pressure at 0.5 cc. volume, so that its pressure is multiplied 100-fold. Thus 1 volume per cent of nitrogen present as impurity in oxygen can be determined with an accuracy approaching 0.001 volume per cent.

In all the methods outlined in the present series of papers, the manometric apparatus described by Van Slyke and Neill (1924) and Van Slyke (1926-27) is used without alteration. The chamber could be modified, so that readings could be taken with the gas at other volumes than the 0.5, 2, and 50 cc. provided by the original chamber, but such modifications have as yet not been found necessary. The same apparatus, used for determination of the blood gases and various other substances by methods previously published in this *Journal*, can also be used without alteration for the gas analyses described in this and the succeeding papers.

Methods A and B described in this paper for determination of O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub> in air by simple absorption are capable of being carried out with errors not exceeding 0.1 volume per cent, and usually within 0.05 volume per cent. When greater accuracy is required, the isolation method is used for CO<sub>2</sub> (Van Slyke, Sendroy, and Liu, 1932) and the combustion method for O<sub>2</sub> (Van Slyke and Hanke, 1932), as described in accompanying papers of this series. Method C, described in this paper for small amounts of N<sub>2</sub> or other inert gas present in oxygen or carbon dioxide, is of the highly accurate type mentioned above.

#### GENERAL POINTS OF TECHNIQUE FOR APPLICATION OF MANOMETRIC APPARATUS TO ANALYSES OF GAS MIXTURES

*Extension of Manometer Scale for Low p<sub>0</sub> Readings*—The zero readings with the mercury in the chamber at the 50 cc. mark fall in the low part of the manometer tube opposite the bottom of the chamber. Most of the closed tube manometers made for the Van Slyke-Neill apparatus before 1931 have scales which fail by 10 or 20 mm. to extend so low, since none of the methods used prior to this time involved zero readings with more than 2 cc. of gas space in the chamber. It is, however, not difficult to improvise an extension of the scale to make possible zero readings at the lower point. On transparent paper a ladder of parallel lines 1 mm. apart is made with black India ink. Each line is made long enough to extend half way around the manometer tube, except that

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every fifth line is made a little longer to facilitate counting the distances. The strip of paper with the lines is cut of sufficient width to extend two-thirds of the way around the manometer tube. It is then pasted onto the tube in such a way that the open third is towards the observer's eye, and the uppermost mark on the paper covers the zero mark on the glass scale. If such a scale extension is used, a convenient way to employ it in connection with the scale already present is to add 100 mm. to each reading made on the latter, and consider the zero point to be the point on the extrapolated scale 100 mm. lower than the original zero of the glass scale.

### *Temperature Control and Corrections*

*Control*—In so far as atmospheric conditions are concerned, the manometric apparatus with the closed manometer is independent of changes in barometric pressure, because the manometer and chamber of the apparatus form a closed system.

Temperature changes, however, occurring in the manometric apparatus in the course of an analysis, cannot be automatically compensated by a device like the thermobarometer. The manometric apparatus is therefore used under conditions to minimize temperature changes during the short time required for an analysis. If the temperature registered by the thermometer in the water jacket of the chamber of the apparatus differs from that registered by a thermometer in the air of the room at the same level, the water jacket is warmed or cooled by wrapping it for a minute or longer in a towel wet with hot or cold water. The chamber is then shaken and the temperature on its thermometer noted. It is desirable to bring it within 0.2° or 0.3° of the room temperature. During an analysis the room temperature is kept as constant as possible.

*Corrections*—If significant temperature changes do occur during an analysis, corrections for them must be applied. The corrections are estimated from the following considerations.

The procedure in most of the analyses is to measure the sample by its pressure,  $P_s$ , as described below for "Measurement of sample," then to remove the gas determined, and finally to measure the pressure,  $P_R$ , of the residual gas. The constituent gas determined is measured by the difference,  $P_s - P_R$ .

$P_S$  of the sample is measured as the difference between two manometer readings,  $p_0$  taken with the manometer chamber empty, and  $p_1$  taken after admission of the sample. These two readings are taken within such a short time interval that there is ordinarily no significant temperature change between them. Similarly  $P_R$  is measured as the difference between two quickly succeeding manometer readings, one before and one after the residual gases have been ejected from the chamber.

Between the observations of  $P_S$  and  $P_R$ , however, a period of several minutes may elapse during which the constituent gas determined is in process of removal by absorption or combustion, so that temperature changes of  $0.2^\circ$  or  $0.3^\circ$  may occur between the  $P_S$  and  $P_R$  measurements. For comparison with  $P_S$  in the calculation of results,  $P_R$  must in such a case be corrected, in order to obtain the value it would have at the temperature of the sample measurement. For this purpose the observed value of  $P_R$  is multiplied by the factor,  $\frac{T_S}{T_R}$ , where  $T_S$  represents the absolute temperature (centigrade +273°) observed in the water jacket of the chamber at the time of the sample measurement, and  $T_R$  is the absolute temperature of the final  $P_R$  measurement. The correction amounts to about 1 part per 3000 for  $0.1^\circ$  temperature change.

$$(1) \quad P_R \text{ corrected to } T_S = P_R \text{ observed} \times \frac{T_S}{T_R}$$

The following values of the factor  $\frac{T_S}{T_R}$  are used for the conditions encountered and are convenient to have at hand. When  $T_S$  is less than  $T_R$ , the factor  $\frac{T_S}{T_R}$  has the value 0.9997 for  $0.1^\circ$  difference between  $T_S$  and  $T_R$ , 0.9993 for  $0.2^\circ$ , 0.9990 for  $0.3^\circ$ , and 0.9986 for  $0.4^\circ$ . When  $T_S$  exceeds  $T_R$  the values of the factor are 1.0003 for  $0.1^\circ$  difference, 1.0007 for  $0.2^\circ$ , 1.0010 for  $0.3^\circ$ , and 1.0014 for  $0.4^\circ$ .

In the analyses described in this paper,  $P_R$  is represented by the pressure,  $P_{N_2}$ , of the residual nitrogen left after absorption of the  $CO_2$  and  $O_2$ .

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The above correction is the only one that need ordinarily concern the analyst who uses the methods described in this series of papers. The precautions outlined under "Control" are usually sufficient, as stated above, to prevent significant temperature changes in the interval between the two successive manometer readings involved in the measurement of any one gas portion.

### *Technique of Manometer Readings*

Before every manometer reading in these gas analyses, the mercury meniscus in the chamber is lowered below either the 2 or the 50 cc. mark, and is then brought slowly up to the mark by admitting mercury from the leveling bulb while the latter rests level with the bottom of the chamber. While the mercury in the chamber is rising to the mark it is observed with a magnifying glass (a good reading glass serves well), and the cock from the leveling bulb is closed just as the top of the curved mercury meniscus reaches the mark. With practice one can bring the mercury in the chamber thus to the same level with a constancy of 0.1 mm. It is essential that the mercury surface should always be brought to the mark in the same manner from below upwards, and never from above downwards, because the manometer readings obtained after these two different approaches differ, slightly but measurably. After the mercury is at the mark in the chamber the reading on the manometer is taken. If there is any doubt concerning the accuracy of the placing of the mercury meniscus in the chamber, the mercury is lowered and brought up to the mark again, and the reading is repeated. In such duplicate observations the readings on the manometer (also with the help of a lens) should differ by not more than 0.1 mm.

### *Preparation of Manometric Chamber to Receive Gas Sample*

The manometric chamber is washed, if necessary, to free it from interfering solutions used in preceding analyses. Then all the water present is removed except the invisible film adherent to the walls. To remove excess water the mercury in the chamber is lowered to the bottom, and then is permitted to rise slowly to the top. The water which collects on the surface is expelled, and about 2 cc. of mercury are driven up into the cup above the chamber. The chamber is then evacuated and the mercury lowered to

the 50 cc. mark. If more water then collects on the mercury surface than will form a slight ring about the edge, the above procedure is repeated to finish the removal of the water. The repetition will not be necessary unless during the first removal the mercury was allowed at some point to rise too rapidly to permit the water to detach itself completely from the glass walls ahead of the ascending mercury.

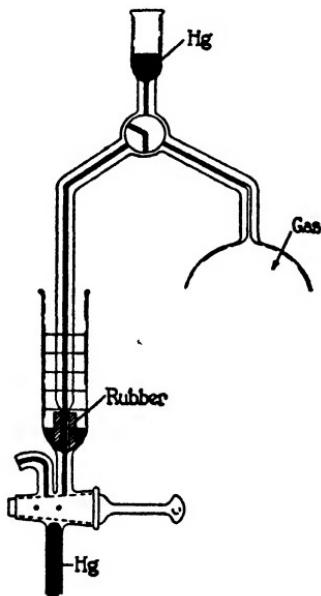


FIG. 1. Admission of gas sample into manometric chamber

The *zero reading*,  $p_0$ , of the apparatus, with the chamber free of gas and visible water, is now taken, as described above under "Technique of manometer readings," with the mercury meniscus at either the 2 or the 50 cc. mark, whichever is to be used in the analysis.

The tip of the capillary from the sample container is inserted into mercury in the cup of the chamber, as shown in Fig. 1. Any satisfactory container with a 3-way cock may be used, such as the familiar Barcroft tube, or the modified Hempel pipette of Van Slyke and Hiller (1928). The connecting capillary should be

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narrowed at the tip, which is fitted with a rubber ring. Such a ring is made by cutting a section about 10 mm. long from a soft rubber tube of about 1 mm. bore with walls 2 mm. thick. The bottom of the ring is beveled slightly by grinding on an emery wheel or with sandpaper, so that it will fit the curvature of the bottom of the cup when pressed into the latter as shown in Fig. 1.

The manometric leveling bulb is first placed in the ring above the chamber, and mercury is forced up through the connecting capillary and 3-way cock of the sample container, displacing the air in its capillary, as shown in Fig. 1. During this operation the capillary is pressed into the bottom of the cup with one hand, so that the rubber ring makes a tight seal under the mercury, while the cocks are turned with the other hand.

### *Admission of Sample Regulated by Volume*

For most gas analyses one takes a sample sufficient to give 450 to 550 mm. of pressure at either 2 or 50 cc. volume, according to whether a micro or macro analysis is to be made. Such a sample is of about 1.5 or 35 cc. volume at atmospheric pressure. Its admission can be regulated with sufficient accuracy as a rule by the following procedure.

The gas container and manometric chamber are arranged as shown in Fig. 1, and the leveling bulb of the chamber is placed about level with the bottom of the chamber. The cock connecting leveling bulb and chamber is left open, so that the contents of the chamber are under slight negative pressure. Gas from the sample container is then admitted to the chamber until the volume in the latter is approximately 1.5 or 35 cc. The volume can, with slight practice, be estimated with sufficient accuracy from the height of the mercury surface in the chamber above the 2 or 50 cc. mark.

Another procedure, which can be used also for samples of size not easily estimated from the marks on the chamber, is to admit the gas sample first to a container which holds at atmospheric pressure approximately the desired amount. From the container the sample is transferred to the manometric chamber by the technique shown in Fig. 9 of Van Slyke and Neill (1924). The water there shown in the container is here replaced by gas.

*Admission of Sample Regulated by Pressure*

For admission of samples intermediate between the 1 to 1.5 and the 30 to 35 cc. sizes the following procedure can be used. It requires somewhat more skill than admission regulated by volume, but has the advantage that it obviates the use of a special container for preliminary approximate measurement of the sample.

The apparatus is prepared and the  $p_0$  reading taken as described above. The sample container is then permitted to hang in position, with its cock closed and the tip of its capillary outlet immersed in the mercury of the cup of the chamber. The capillary of the gas container is filled with mercury as in Fig. 1. The mercury in the chamber is then lowered till it is about 10 mm. above the 50 cc. mark. The cock between leveling bulb and chamber is then closed, and the leveling bulb is rested in the medium position, level with the bottom of the evacuated chamber. The tip of the capillary from the sample container is then pressed into the bottom of the cup, as shown in Fig. 1, and the cock of the container is turned to connect container and chamber. Then the cock at the top of the chamber is slightly opened, just long enough to admit enough gas to depress the mercury level in the chamber a little below the 50 cc. mark. The mercury in the manometer rises 20 to 40 mm. The cock leading to the manometric leveling bulb is now opened to admit enough mercury to the chamber to make the meniscus rise again about 10 mm. above the 50 cc. mark; then enough gas is again let in to force the mercury a little below the mark. This is repeated one, two, or three times, until the mercury in the manometer has risen to about the desired final level. During this procedure one holds the capillary tube of the sample container in position with the left hand, while with the right hand one alternately opens the cock at the top of the chamber to admit gas, then the cock from the leveling bulb to admit mercury. The procedure is in fact simple, and the admission of the entire sample by alternate turns of the two cocks requires less than a minute. Before the last admission of gas, the amount of mercury admitted is regulated from previous experience so that the subsequent admission of enough gas to press the mercury down to the mark will raise the mercury column in the manometer to the desired height; e.g., the amount of mercury admitted before the last portion of

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gas may need to be only enough to raise the meniscus 5 mm., instead of 10 or 15, above the 50 cc. mark in the chamber.

### *Measurement of Sample*

After the sample has been admitted by either of the above procedures, the sample container is removed and the bore of the cock at the top of the chamber is filled with mercury from the cup (this cock is gas-tight only when *both bores are filled with mercury*). The mercury meniscus is then lowered below either the 2 cc. or the 50 cc. mark, and is brought back to the mark as above directed. The manometer reading  $p_1$  is then taken, and the temperature of the chamber is read on the thermometer in the water jacket. The size of the gas sample is indicated by the pressure  $P_S$ , which the gas exerts at the chosen volume, either 2 or 50 cc.

$$(2) \quad P_S = p_1 - p_0$$

The *volume*,  $V_S$ , of the gas sample, reduced to 0°, 760 mm., can be calculated from  $P_S$  by the formula:

$$(3) \quad V_S = a \times \frac{P_S}{760} \times \frac{1}{1 + 0.00384t}$$

Here  $a$  is the volume (50 or 2 cc., as a rule) of the gas sample when its pressure,  $P_S$ , is measured, and  $t$  is the temperature centigrade. The formula is similar to the familiar one

$$V_{0^\circ, 760} = V_{t^\circ, B-W \text{ mm.}} \times \frac{B - W}{760 (1 + 0.00367t)}$$

In Equation 3,  $a$  represents the observed volume at which the gas is measured under  $P_S$ , instead of  $B - W$ , mm. of pressure. The coefficient 0.00384 for  $t$ , instead of the usual 0.00367, is employed in order to correct for expansion of mercury in the manometer with temperature, as explained on p. 540 of Van Slyke and Neill's paper (1924).

In calculating the results of analyses described in this paper only pressure figures are used, so that transformation of the sample measurement into terms of volume is not necessary. Equation 3 is given, however, as it is sometimes desirable to measure a gas

by pressure in the apparatus, and then to calculate the volume under standard conditions for other purposes.

#### METHOD A. DETERMINATION OF OXYGEN AND CARBON DIOXIDE IN AIR

The gas sample is measured by the pressure it exerts at 50 cc. volume, and the diminutions of pressure noted after successive absorption of CO<sub>2</sub> in the chamber with sodium hydroxide, and of oxygen with hyposulfite, are used to calculate these gases. For CO<sub>2</sub>, when it forms less than 10 per cent of the gas analyzed, this method is less accurate than the isolation procedure described in the second paper of this series (Van Slyke, Sendroy, and Liu, 1932), and for O<sub>2</sub> the precision is less than in the method based on combustion with H<sub>2</sub> given in Paper IV (Van Slyke and Hanke, 1932). The determinations by simple absorption are, however, most convenient and rapid, and are reliable within limits which are sufficiently precise for many purposes.

Gas samples are taken of 30 to 35 cc. volume, giving a pressure at 50 cc. of about 500 mm. of mercury. The manometer readings are reproducible to 0.2 mm., corresponding to 0.04 volume per cent of the sample. If errors of this size, but opposite in direction, were made before and after absorption of either gas, the resultant error in the analysis would be the sum, or 0.08 per cent of an atmosphere. However, by performing check readings for each observation of the manometer, it is possible to keep the usual error within  $\pm 0.05$  volume per cent (see Table I).

#### Reagents

*1 N Sodium Hydroxide*—The solution need not be freed of air for this analysis.

*30 Per Cent Sodium Hyposulfite in 2 N Potassium Hydroxide*—15 gm. of the hyposulfite, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, are stirred up with 50 cc. of 2 N potassium hydroxide solution and filtered quickly through cotton into a 100 cc. flask, containing enough paraffin oil to make a layer 1 or 2 cm. thick.

Fieser's anthrahydroquinone- $\beta$ -sulfonate catalyst, which has proved its convenience in blood oxygen determinations (Van Slyke, 1927), is here left out of the hyposulfite solution, because the intensely red color of the catalyst would prevent reading the

mercury meniscus when the solution is in the chamber. The analysis could be accomplished in 1 minute less time with the sulfonate catalyst present, but it appears usually to be preferable to use the water-clear solution of hyposulfite without the catalyst.

For Methods A and B the solution is used without freeing it of dissolved air, because the oxygen is absorbed with the system at nearly atmospheric pressure. For Method C, however, the solution is freed of air as described on p. 535 of Van Slyke and Neill (1924). The air-free solution is kept over mercury and out of contact with air. A layer of oil would not protect it from absorption of sufficient atmospheric nitrogen to affect the results of analyses by Method C.

#### *Measurement of Sample*

The sample of 30 to 35 cc. is admitted into the apparatus as described above on p. 516, and  $P_S$  is measured as described on p. 518 for "Measurement of sample."

$$P_S = p_1 - p_0$$

#### *Absorption of CO<sub>2</sub>*

1 cc. of 1 N sodium hydroxide is measured accurately into the chamber from a stop-cock pipette in the manner shown in Fig. 3, p. 125, of a previous paper (Van Slyke, 1927). During the admission of the alkali the manometric leveling bulb is at the medium level, with the mercury surface in the bulb at about the height of the 50 cc. mark on the chamber. The cock between leveling bulb and chamber is left open, so that the contents of the chamber are under slightly negative pressure, and the chamber is about one-third full of mercury. After the alkali is admitted the chamber is shaken for 2 minutes so that the alkali will absorb the CO<sub>2</sub> from the gas sample. On account of the weight of mercury in the chamber the shaking is somewhat slower than in most analyses with this apparatus. During the shaking, the cock between the chamber and its leveling bulb is left open. The 10 or 15 cc. of mercury in the chamber and the alkali solution over the mercury are thrown about in such a way that thorough contact between gas and liquid is obtained, and CO<sub>2</sub> absorption is completed in 2 minutes. It is usually, in fact, finished during the 1st minute.

After absorption is finished the manometer reading  $p_2$  is taken with the meniscus of the *mercury* (not of the water solution) at the 50 cc. mark in the chamber.

### *Absorption of Oxygen with Hyposulfite*

After the  $p_2$  reading has been taken the cock between the leveling bulb and the chamber is opened and 3.00 cc. of hyposulfite solution, accurately measured from a calibrated stop-cock pipette, are run into the chamber in the same manner in which the 1 cc. of sodium hydroxide was added. The absorption of oxygen is accomplished by shaking the chamber in the same manner as for  $\text{CO}_2$  absorption, except that for oxygen 3 minutes instead of 2 are taken.

After the absorption is completed the meniscus of the *mercury* is again brought to the 50 cc. mark and  $p_3$  is read.

The gas is then ejected from the top of the chamber without loss of any of the solution (Van Slyke, 1926-27, p. 240) and  $p_4$  is read with the 4 cc. of solution, but with no gas, in the chamber, and with the mercury meniscus again at the 50 cc. mark. The analysis is now complete.

### *Calculation*

All quantities of gas measured are calculated in terms of the pressure exerted with the gas at 50 cc. volume and at the temperature of the  $P_S$  measurement. The sample is calculated as:

$$P_S = p_1 - p_0$$

The pressure of  $\text{O}_2 + \text{N}_2$  is measured at 49 cc. volume. To calculate it for 50 cc. volume, therefore, it is necessary to multiply the observed pressure by  $\frac{50}{49}$ , or 0.98.

$$(4) \quad P_{\text{O}_2 + \text{N}_2} = 0.98 (p_2 - [p_0 + c])$$

The significance of the  $c$  correction will be discussed below.

The pressure of the  $\text{CO}_2$  is calculated by subtracting the pressure of the  $\text{O}_2 + \text{N}_2$  from that of the total sample.

$$(5) \quad P_{\text{CO}_2} = P_S - P_{\text{O}_2 + \text{N}_2}$$

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The pressure of the N<sub>2</sub> at 46 cc. volume is measured as  $p_3 - p_4$ . At 50 cc. therefore one calculates:

$$(6) \quad P_{N_2} = 0.92 (p_3 - p_4)$$

The oxygen is calculated by subtracting the N<sub>2</sub> from the O<sub>2</sub> + N<sub>2</sub>.

$$(7) \quad P_{O_2} = P_{O_2 + N_2} - P_{N_2}$$

The final results are calculated from the above data as follows:

$$(8) \quad \text{Per cent CO}_2 = \frac{100 P_{CO_2}}{P_S}$$

$$(9) \quad \text{Per cent O}_2 = \frac{100 P_{O_2}}{P_S}$$

$$(10) \quad \text{Per cent N}_2 = \frac{100 P_{N_2}}{P_S}$$

### Remarks on Calculation

As shown above, one must multiply the observed O<sub>2</sub>+N<sub>2</sub> and N<sub>2</sub> pressures by 0.98 and 0.92, respectively, in order to calculate the pressures at 50 cc. volume from those at 49 and 46 cc. In practice, the simplest way to make this calculation is to subtract 0.02 and 0.08 of their values from the observed pressures,  $p_2 - [p_0 + c]$ , and  $p_3 - p_4$ .

*Example of Calculation*—The data are from an analysis of laboratory air.  $c = 1.4$  mm. for the chamber used.

$$p_1 = 551.4 \qquad \qquad \qquad p_2 = 561.8$$

$$p_0 = \underline{88.0} \qquad \qquad \qquad p_0 + c = \underline{89.4}$$

$$P_S = 463.4 \qquad P_{O_2 + N_2} \text{ at } 49 \text{ cc.} = 472.4$$

$$0.02 \text{ of same} = \underline{9.4}$$

$$P_{O_2 + N_2} \text{ at } 50 \text{ cc.} = 463.0$$

$$p_2 = \underline{487.0}$$

$$p_4 = \underline{89.0}$$

$$P_{N_2} \text{ at } 46 \text{ cc.} = 398.0$$

$$0.08 \text{ of same} = \underline{31.8}$$

$$P_{N_2} \text{ at } 50 \text{ cc.} = 366.2$$

$$\text{Per cent CO}_2 = 100 \times \frac{463.4 - 463.0}{463.4} = 0.09$$

$$\text{Per cent O}_2 = 100 \times \frac{463.0 - 366.2}{463.4} = 20.89$$

$$\text{Per cent N}_2 = 100 \times \frac{366.2}{463.4} = 79.02$$

### *Determining the c Correction*

. After the CO<sub>2</sub> has been absorbed the pressure exerted by the residual O<sub>2</sub> + N<sub>2</sub> at 49 cc. volume would be inexactly calculated as  $p_2 - p_0$ . The manometer reading without any gas present, but with the 1 cc. of NaOH solution in the chamber, would be slightly *higher* than the  $p_0$  observed at the beginning of the analysis, with neither gas nor solution in the chamber. The weight of the short column of solution in the chamber presses on the mercury there, and in consequence raises the height of the mercury column in the manometer tube required to balance the meniscus in the chamber at the 50 cc. mark. The *c* correction required for this effect varies somewhat with the shape of the bottom of the chamber and the consequent height of the column of 1 cc. of solution. The value of *c* is, however, usually in the neighborhood of 1.5 mm.

The *c* correction is determined as follows: The  $p_0$  point is determined, with the chamber free of both gas and visible amounts of water, as described on p. 515. Then 1 cc. of 1 N sodium hydroxide is admitted, as described for absorption of CO<sub>2</sub>, the chamber is evacuated till the mercury falls to the 50 cc. mark, and the air is extracted from the solution by shaking the latter 1 minute. The extracted small bubble of gas is ejected from the top of the chamber without loss of solution. The chamber is again

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evacuated and the mercury meniscus is brought to the 50 cc. mark. The manometer is again read. This reading is  $p_0 + c$ .

The difference between it and the  $p_0$  reading is the  $c$  correction. To determine  $c$  within 0.1 mm. one makes several check readings of  $p_0$  with the chamber empty and also several of  $p_0 + c$ , with the 1 cc. of solution in the chamber. Once determined, the  $c$  correction serves for all analyses in which the same chamber is used.

### Temperature Correction

If, during the interval between the  $P_S$  and the  $P_{O_2 + N_2}$  or  $P_{N_2}$  measurement, changes exceeding 0.1° from the temperature of the  $P_S$  measurement are noted in the thermometer of the water jacket, the value of  $P_{O_2 + N_2}$  or  $P_{N_2}$  is calculated by Equation 10 or 11 instead of by Equation 4 or 6.

$$(11) \quad \text{Corrected } P_{O_2 + N_2} = 0.98 (p_2 - [p_0 + c] + 1.3 [t_0 - t_2]) \times \frac{T_0}{T_2}$$

$$(12) \quad \text{Corrected } P_{N_2} = 0.92 (p_2 - p_4) \times \frac{T_0}{T_4}$$

$T_0$ ,  $T_2$ , and  $T_4$  = values of absolute temperature at the readings  $p_0$ ,  $p_2$ , and  $p_4$ ;  $t_0$  and  $t_2$  = temperature in degrees centigrade at readings  $p_0$  and  $p_2$ . The values of the ratio,  $T_0:T_2$ , or  $T_0:T_4$ , for ordinary temperature changes are given below Equation 1 on p. 513. In Equation 11 the term,  $1.3 (t_0 - t_2)$ , serves to correct for the vapor pressure change, of about 1.3 mm. per 1°, caused by the temperature change,  $t_0 - t_2$ . Equation 11 covers the temperature correction in an exceptional case, where an intermediate operation (absorption of CO<sub>2</sub>) may allow time for a change of temperature between the two readings ( $p_0$  and  $p_2$ ) on which a single gas measurement ( $P_{O_2 + N_2}$ ) depends.

### Necessity for Accuracy in Measurement of Volumes of Alkali and Hyposulfite Solutions Added

The accuracy with which the volumes 49 and 46 cc. are defined, at which the pressures of residual gases are measured after absorption of CO<sub>2</sub> and O<sub>2</sub> respectively, is determined by the accuracy

with which the 1 and 3 cc. portions of alkali and hyposulfite solution, respectively, are measured into the chamber. An error of 0.01 cc. in the measurement of either solution would cause an error of 1 part in 5000 in the volume of gas space at which the pressure is measured. With calibrated stop-cock pipettes, however, it is simple to make the deliveries of solution into the chamber with errors less than 0.01 cc.

TABLE I  
*Manometric Analysis of Gas Mixtures for Carbon Dioxide, Oxygen, and Nitrogen by Simple Absorption*  
 Pressure measurements at 50 cc. volume

Sample No.	Manometric analysis by Method A with approximately 35 cc. samples			Haldane analysis, with approximately 10 cc. samples		
	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>
1	per cent	per cent	per cent	per cent	per cent	per cent
	5.59	19.68	74.73	5.55	19.72	74.73
	5.53	19.75	74.72	5.57	19.70	74.73
	5.49	19.70	74.81			
	5.59	19.66	74.75			
2	10.36			10.42		
	10.45			10.43		
3	6.66	19.46	73.88	6.70	19.46	73.84
	6.70	19.38	73.92	6.71	19.49	73.80
4	3.98	19.91	76.11	4.01	19.93	76.06
	3.88	19.96	76.16	4.00	19.98	76.02
	3.95	20.07	75.98			

Similar accuracy in the calibration of the exact total gas volume held above the 50 cc. mark is not necessary. Any error in this calibration will have so nearly a proportional effect on pressure measurements at 49 and 46 cc., that an error of 0.1 cc. in the 50 cc. calibration is required to affect oxygen results by 1 part in 5000.

Table I shows results obtained with the technique outlined above

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compared with those yielded by the standard Haldane (1912) method for air analysis.

### METHOD B. NITROGEN IN AIR, OR IN MIXTURES OF CO<sub>2</sub>, O<sub>2</sub>, AND N<sub>2</sub> WITH OVER 10 PER CENT OF N<sub>2</sub>

In some cases, as in the lung volume determinations by the nitrogen dilution method used by Lundsgaard and Van Slyke

TABLE II

*Determination of Nitrogen in Atmospheric Air and in Air Diluted with Oxygen. Simple Absorption of Carbon Dioxide and Oxygen by Method B*

Pressure measurements at 50 cc. volume

Sam- ple No.	Observations						Calculations			
	$p_0$ Cham- ber empty	$p_1$ Sample in cham- ber	Tem- pera- ture at $P_S$	$\frac{p_2}{N_2 + Na_2S_2O_4}$ in cham- ber	$p_3$ $Na_2S_2O_4$ only in cham- ber	Tem- pera- ture at $P_{N_2}$	$P_S =$ $p_1 - p_0$	$P_{N_2}$	Per cent $N_2 = 100$ $\times \frac{P_{N_2}}{P_S}$	
	mm.	mm.	°C.	mm.	mm.	°C.	mm.	mm.	mm.	
1	89.5	609.7	26.5	526.0	89.0	26.7	520.2	410.7	410.5	78.91*
2	90.6	617.2	27.2	533.1	90.1	27.4	526.6	416.3	416.1	78.92*
3	88.6	617.2	26.4	532.6	88.2	26.6	528.4	417.8	417.6	79.03*
4	87.0	624.0	24.5	538.4	86.7	24.7	537.0	424.7	424.5	79.05*
5	85.7	617.0	25.0	531.6	84.9	25.2	531.3	419.8	419.6	79.01*
6	85.4	487.8	25.0	239.7	84.7	25.2	402.4	145.6	145.5	36.13
	85.4	488.4	25.2	240.0	84.6	25.4	403.0	146.0	145.9	36.21
7	84.4	497.8	24.0	205.2	83.9	24.2	413.4	114.0	113.9	27.53
	84.6	494.3	24.6	204.4	84.5	24.9	409.7	112.6	112.5	27.47
8	85.7	496.8	24.9	233.0	84.6	25.1	411.1	139.4	139.3	33.90
	85.2	487.5	25.0	229.8	84.7	25.2	402.3	136.4	136.3	33.90
9	84.3	498.8	24.1	201.4	83.5	24.3	414.5	110.8	110.7	26.72
	84.0	486.9	23.7	197.9	83.3	24.0	402.9	107.7	107.6	26.72

\* Atmospheric air. Nitrogen content = 79.04 per cent.

(1918), the nitrogen content of the air is the only figure desired. In this case the analysis is simplified by absorbing the CO<sub>2</sub> and O<sub>2</sub> together by the alkaline hyposulfite.

The sample is measured as in the preceding analysis.

$$p_1 - p_0$$

Then 3 cc. of the hyposulfite solution are introduced into the chamber, as above described, and the O<sub>2</sub> and CO<sub>2</sub> are absorbed together by 3 minutes shaking. Reading  $p_2$  is taken with the mercury meniscus at the 50 cc. mark. The gases are then ejected without loss of solution and reading  $p_s$  is taken.

All readings being taken with the *mercury* meniscus in the chamber at the 50 cc. mark, the calculations resemble those of the preceding analysis.

$$P_{N_2} = 0.94 (p_s - p_2)$$

$$\text{Per cent N}_2 = 100 \times \frac{P_{N_2}}{P_s}$$

$$\text{Per cent (CO}_2 + \text{O}_2) = 100 - \text{per cent N}_2$$

Table II indicates the results for N<sub>2</sub> obtained by the above method. Samples 1 to 5 were those of atmospheric air, which should contain 79.04 per cent of N<sub>2</sub>. Samples 6 to 9 were mixtures of respired air and oxygen analyzed in the course of lung volume determinations.

#### METHOD C. SMALL AMOUNTS OF N<sub>2</sub> OR OTHER INERT GAS PRESENT AS IMPURITY IN O<sub>2</sub> OR CO<sub>2</sub>

In case one wishes to determine traces of N<sub>2</sub> as impurity in CO<sub>2</sub> or O<sub>2</sub>, the unabsorbed N<sub>2</sub> is measured by its pressure at 2 or 0.5 cc. volume. *In such analyses it is necessary to use gas-free hyposulfite solution kept over mercury*, because when the oxygen has been absorbed the contents of the chamber are under a high vacuum, and dissolved N<sub>2</sub> present in the hyposulfite solution would escape into the gas phase in sufficient amount to affect the results.  $P_s$  is measured as  $p_1 - p_0$ , in the manner described above. Then the O<sub>2</sub> and CO<sub>2</sub> are absorbed with air-free hyposulfite, and  $p_2$  is read with the unabsorbed gas (*solution* meniscus) at either 2 or 0.5 cc. volume. The gas is then ejected without loss of solution and  $p_s$  is read, with the chamber free of gas and the solution meniscus at the same mark as for the  $p_2$  reading.

$$P_{N_2} = p_2 - p_s$$

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$$\text{Per cent N}_2 = 100 \times \frac{a}{A} \times \frac{P_{N_2}}{P_S}$$

$a$  is the small volume, 0.5 or 2 cc., at which  $P_{N_2}$  is measured, and  $A$  is the total chamber volume, 50 cc. in the usual chamber, at which  $P_S$  is measured. For chambers of the ordinary dimensions,

TABLE III  
*Determination of Nitrogen (or Hydrogen) as an Impurity in Oxygen by Method C. P<sub>S</sub> at 50 Cc.*

Tank No.	Observations								Calculations				Formula for calculation of per cent N <sub>2</sub>
	$p_0$ Chamber empty mm.	$p_0$ Sample in chamber mm.	Temperature at $P_S$ measurement °C.	$p_2$ $N_2 + Na_2S_2O_3$ in chamber mm.	$p_2$ Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> only in chamber mm.	Temperature at $P_{N_2}$ measurement °C	Gas volume $a$ at $P_{N_2}$ measurement cc.	$P_S = p_1 - p_0$ mm.	$P_{N_2}$ = $p_2 - p_1$ uncorrected mm.	Corrected for temper- ature change from $P_S$ mm.	Per cent N <sub>2</sub>		
1	75.6	479.8	21.8	305.3	149.1	22.4	0.5	404.2	156.2	155.9	0.386	$\frac{P_{N_2}}{P_S}$	
	76.9	307.4	22.8	241.4	150.9	23.2	0.5	230.5	90.5	90.4	0.392		
2	77.6	485.8	23.2	326.5	150.5	24.0	0.5	408.2	176.0	175.5	0.430	“	
	78.4	482.8	24.0	325.3	150.3	24.5	0.5	404.4	175.0	174.7	0.432		
3	89.1	487.0	24.0	229.6	172.8	24.4	2.0	397.9	56.8	56.7	0.570	$\frac{4 P_{N_2}}{P_S}$	
	89.7	485.7	24.4	227.4	172.0	24.9	2.0	396.0	55.4	55.3	0.559		

with  $A = 50$  cc. and  $a = 0.5$  or 2 cc., the calculations simplify to the following.

$$\text{Per cent N}_2 = \frac{N_2}{S} \text{ when } a = 0.5 \text{ cc. or}$$

$$\text{Per cent N}_2 = \frac{4 P}{S} \text{ when } a = 2 \text{ cc.}^1$$

<sup>1</sup> If, on checking the calibration of  $a$  with water,  $a'$  the actual volume is found to be different from the supposed gas volume (0.5 or 2 cc.), the observed pressure  $P_{N_2}$  must here be corrected by multiplication by  $\frac{a'}{a}$ .

The gas thus calculated as "N<sub>2</sub>" may be N<sub>2</sub>, H<sub>2</sub>, or any other gas which does not combine with alkali or hyposulfite.

Table III indicates the results obtained when the contents of three tanks of commercial oxygen gas were thus analyzed for inert impurities.

#### SUMMARY

Methods are described for the determination of carbon dioxide, oxygen, and nitrogen in gas mixtures by simple absorption in the manometric apparatus originally designed by Van Slyke and Neill for blood gases.

The gas sample and the nitrogen are measured by the pressures they exert at defined volumes. Carbon dioxide and oxygen are measured by successively absorbing them with alkali and hyposulfite solution, respectively, in the chamber of the manometric apparatus, and noting the resultant decreases in pressure.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### II. CARBON DIOXIDE BY THE ISOLATION METHOD

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In this paper there is described a procedure whereby the CO<sub>2</sub> in a gas mixture is first isolated from other gases by absorption with alkali solution in the chamber of the manometric apparatus. The other gases are then ejected, the absorbed CO<sub>2</sub> is set free by acid, and is determined as in blood analyses. By this procedure the CO<sub>2</sub> in any desired volume of gas can be absorbed, and then set free and determined by the pressure it exerts at 2 or 0.5 cc. volume. The error with the usual manometric chamber, is, as in blood gas determination, less than 1 per cent of the amount of CO<sub>2</sub> determined, and could doubtless be further reduced by modifying the manometric chamber. Thus Van Slyke, Hastings, Heidelberger, and Neill (1922), using a chamber of 100 instead of 50 cc. total volume, with measurements of CO<sub>2</sub> pressures at 5 instead of 2 cc. volume, limited the error of blood CO<sub>2</sub> determinations to about 1 part in 500. The analyses published in this paper were obtained with the usual 50 cc. chamber, and therefore do not represent the maximum accuracy that could be obtained by modifying the chamber for that purpose.

A carbon dioxide method in which, as in this, the error in terms of per cent of the total gas mixture diminishes in proportion to the CO<sub>2</sub> content, is especially adapted to analysis of gas mixtures containing small amounts of carbon dioxide. With the isolation method, and the usual 50 cc. manometric chamber, one can readily determine the CO<sub>2</sub> content of atmospheric air to 0.0003 volume

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per cent, or 0.01 of the amount present. Such precision with apparatus modeled on the usual principles of gas analysis can be obtained only with most elaborate precautions, because it would involve measurement of total gas volumes with an accuracy of 1 part in 300,000.

The method serves well for determination of carbon dioxide in expired or alveolar air, where the CO<sub>2</sub> content runs from 6 to 2 per cent. The error, with the usual manometric chamber employed, is within 1 per cent of the amounts of CO<sub>2</sub> determined.

In analyses of gases with more than 10 or 15 per cent of CO<sub>2</sub>, the simple absorption method, described in the preceding paper (Van Slyke and Sendroy, 1932), is preferable, unless it is desirable to use minimal gas samples. In that case the micro form of the method here described can be used, with samples of only 1 to 1.5 cc.

#### *Reagents*

*5 N Sodium Hydroxide, Approximate.*

*1 N Hydrochloric Acid, Approximate*—83 cc. of concentrated hydrochloric acid of 1.19 specific gravity diluted to a liter.

*0.1 N Sodium Hydroxide, Approximate, of Minimal CO<sub>2</sub> Content*—Sodium hydroxide is dissolved in an equal volume of water and the solution is let stand till the nearly insoluble Na<sub>2</sub>CO<sub>3</sub> has settled. Of the supernatant solution, 6 cc. are pipetted into 1 liter of water, which has been freed of CO<sub>2</sub> by adding a drop of concentrated hydrochloric acid and boiling. About 1 cc. of 1 per cent alizarin red solution is added. The 0.1 N alkali solution is immediately poured into 50 cc. flasks or bottles closed with parafined corks or vaselined glass stoppers. After one of these flasks has been opened to use part of the solution, the residue is thrown away. As an alternative method of preservation larger amounts of the alkali may be kept in closed air-free containers over mercury, as shown in Fig. 6 of Van Slyke and Neill (1924).

#### *Apparatus*

The only special apparatus besides the manometric is a 25 cc. burette for holding CO<sub>2</sub>-free NaOH solution. The tip of the burette is provided with a rubber ring and must be long enough to fit into the cup of the manometric chamber, as shown in Fig. 3 of Van Slyke (1927). The top of the burette is closed by a 1-hole

stopper into which is fitted a soda-lime tube to prevent entrance of atmospheric CO<sub>2</sub>. When the burette is not in use the outlet is kept immersed in mercury to minimize absorption of atmospheric CO<sub>2</sub> by the alkali at the tip.

**METHOD A, FOR GAS SAMPLES NOT EXCEEDING THE VOLUME OF THE MANOMETRIC CHAMBER**

In this method the sample is measured in one portion by the pressure it exerts at 50 or 2 cc. volume in the chamber. It is the method of choice when the gas analyzed contains over 1 (and less than 10) per cent of CO<sub>2</sub>, or when the CO<sub>2</sub> content is less than 1 per cent and maximal accuracy is not essential. When the CO<sub>2</sub> content is less than 1 per cent, Method B, described later in this paper, is more exact, but it is somewhat less rapid.

It is desirable that the analyst familiarize himself with the introductory sections on general technique in Paper I of this series (Van Slyke and Sendroy, 1932).

*Introduction and Measurement of Sample*

*Macro Samples*—Before the gas sample is admitted to the chamber one estimates the approximate pressure in mm. which a sample of desirable size will exert at 50 cc. volume. A desirable sample will contain 0.5 to 1.0 cc. of CO<sub>2</sub>, which will give a pressure of 200 to 400 mm. at 2 cc. volume. A simple rule to follow to calculate the pressure which a sample of desired size will exert at 50 cc. is to divide 1200 by the expected percentage of CO<sub>2</sub> in the gas. E.g., if alveolar air, with probably 6 per cent CO<sub>2</sub>, is analyzed, a sample is taken which will give a  $P_s$  of  $\frac{1200}{6} = 200$  mm. pressure

at 50 cc. The CO<sub>2</sub> in this sample will then exert  $0.06 \times 200 = 12$  mm. pressure at 50 cc., and 25 times as much, or 300 mm. at 2 cc. when  $P_{CO_2}$  is determined in the final measurement. When expired air from a Tissot spirometer, with a CO<sub>2</sub> content of usually about 4 per cent is analyzed, one takes sufficient sample to give a  $P_s$  of about 300 mm. If gas of less than 1.5 per cent CO<sub>2</sub> content is analyzed, as large a sample as possible is taken; enough to give a  $P_s$  of 500 mm.

Before the sample is admitted the chamber is washed with acidulated water, which is ejected by slow admission of mercury.

## II. Manometric CO<sub>2</sub> by Isolation

The  $p_0$  reading is then taken, with the chamber empty of gas and the mercury at the 50 cc. mark. The sample of desired size is then admitted as described for "Admission of sample regulated by pressure" in the preceding paper (Van Slyke and Sendroy, 1932). The  $p_1$  reading is then taken with the mercury meniscus again at the 50 cc. mark.

$$P_S = p_1 - p_0$$

*Micro Samples*—A sample of about 1.5 cc. volume at atmospheric pressure is admitted as described by Van Slyke and Sendroy (1932) for "Admission of sample regulated by volume." The readings of  $p_0$  and  $p_1$  in this case are taken with the mercury in the chamber at the 2 instead of the 50 cc. mark.

### *Absorption of CO<sub>2</sub> from Gas Sample*

After the gas sample has been measured, 3.0 cc. of the CO<sub>2</sub>-free 0.1 N sodium hydroxide solution, measured to 0.1 cc., are admitted into the chamber from the soda-lime guarded burette. The addition of alkali is made in the manner described for "Quantitative transfer of solution to the chamber without washing" in a previous paper (Van Slyke, 1927). Before inserting the burette tip into the cup of the chamber, 0.5 cc. of the solution is wasted, in order to remove from the tip the drop which may have absorbed CO<sub>2</sub> from the air.

After admission of the alkali, the mercury in the chamber is lowered until only the lower third of the chamber is filled with the metal. The chamber is then shaken rather slowly for 2 minutes. This causes complete absorption of the CO<sub>2</sub> by the alkali, which is thrown about on top of the mercury in such a way that it comes into thorough contact with the gas.

The residual gases are then ejected. It is not necessary that the last few c.mm. of gas be ejected, but it is essential that none of the alkali solution rise into the cup. The ejection of gas is therefore stopped when the alkali solution has entered the bore of the stop-cock.

After ejection of unabsorbed gases, 1 cc. of the 1 N hydrochloric acid is placed in the cup, and 0.5 cc. is run into the chamber. The CO<sub>2</sub> is now determined as described for blood analyses (Van Slyke and Neill, 1924). The stop-cock of the chamber is sealed with

mercury and the liberated  $\text{CO}_2$  is extracted by 2 minutes shaking at the 50 cc. mark. The reading  $p_2$  is then taken, with the precautions for this measurement given by Van Slyke and Neill (1924, p. 533).

The reading of the gas pressure  $p_2$  is taken with 2 cc. of gas volume, unless so little  $\text{CO}_2$  is present that the  $P_{\text{CO}_2}$  at 2 cc. volume is less than 100 mm. In this case the reading is taken with the gas at 0.5 cc. volume.

The stop-cock controlling the mercury in the chamber is opened, and the  $\text{CO}_2$  is absorbed with 0.3 cc. of 5 N sodium hydroxide solution, as described on p. 546, of Van Slyke and Neill. Then reading  $p_3$  is taken with the same gas volume as at the  $p_2$  reading. The alizarin indicator serves to show that the entire solution in the chamber turns alkaline.

A blank analysis is performed, in which no gas is admitted to the chamber. The pressure fall observed when the 5 N sodium hydroxide is added is the  $c$  correction. It should not exceed 4 to 6 mm. with the gas at 2 cc. volume if the 0.1 N alkali solution has been prepared and handled with the above outlined precautions to minimize its  $\text{CO}_2$  content.

$$P_{\text{CO}_2} = p_2 - p_3 - c$$

#### *Calculation*

The  $\text{CO}_2$  content of the gas is calculated as:

$$\text{Volumes per cent CO}_2 = \frac{P_{\text{CO}_2}}{P_S} \times \text{factor}$$

The values of the factor are given in Table I.

The factors of Table I are calculated as follows: The volume,  $V_{\text{CO}_2}$ , of  $\text{CO}_2$ , in cc., reduced to 0°, 760 mm., present in the sample, is calculated by multiplying  $P_{\text{CO}_2}$  by the factor,  $f_1$ , which is derived from Equation 4 of Van Slyke and Neill (1924).

$$760 (1 + 0.00384t) \left( 1 + \frac{S \alpha'}{A - S} \right)$$

$A$  = total volume of chamber at lower mark = 50 cc. in present chamber;  $S$  = volume of solution from which the  $\text{CO}_2$  is extracted = 3.5 cc. in this analysis;  $a$  = volume at which the pressure of

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the extracted CO<sub>2</sub> gas is measured = 2 or 0.5 cc.;  $t$  = temperature centigrade;  $\alpha'$  is the distribution coefficient of CO<sub>2</sub> between

TABLE I  
*Factors by Which Ratio,  $P_{CO_2}:P_S$ , Is Multiplied in Order to Calculate Volume Per Cent of CO<sub>2</sub>*

Temperature °C.	Factors when sample pressure is taken at 50 cc. volume		Factors when sample pressure is taken at 2 cc. volume	
	$P_{CO_2}$ measured with gas at 2 cc. volume	$P_{CO_2}$ measured with gas at 0.5 cc. volume	$P_{CO_2}$ measured with gas at 2 cc. volume	$P_{CO_2}$ measured with gas at 0.5 cc. volume
10	4.444	1.132	111.1	28.29
11	32	29	110.8	22
12	21	26	0.5	15
13	11	24	0.3	09
14	02	22	0.0	04
15	4.393	20	109.8	27.98
16	83	17	9.6	92
17	73	15	9.3	87
18	65	13	9.1	82
19	58	12	9.0	77
20	51	10	8.8	72
21	44	08	8.6	68
22	37	06	8.4	63
23	30	04	8.3	59
24	24	02	8.1	54
25	18	00	7.9	50
26	12	1.099	7.8	47
27	06	97	7.6	44
28	00	96	7.5	41
29	4.295	95	7.4	38
30	91	94	7.3	34
31	86	93	7.2	31
32	82	92	7.0	27
33	77	91	6.9	24
34	73	90	6.8	20

the gaseous and aqueous phases;  $i$  = factor correcting for reabsorption of CO<sub>2</sub> while the volume of the extracted gas is being

diminished from  $A - S$  to  $a$  cc. The values of  $i$  used are those found by Van Slyke and Sendroy (1927); *viz.*, 1.017 when  $P_{CO_2}$  is measured at 2 cc. volume, and 1.037 when  $P_{CO_2}$  is measured at 0.5 cc. volume. (Values of 100  $f_1$  are given in Table IV.)

The volume,  $V_S$ , of the sample, in cc. reduced to 0°, 760 mm., is calculated by multiplying  $P_S$  by  $f_2$ .

$$f_2 = 760 (1 + 0.00384t)$$

The symbols,  $a$  and  $t$ , have the same significance as above. The coefficient 0.00384 instead of the usual 0.00367 is used in the denominator in order to correct for expansion of the mercury in the manometer with temperature, as discussed by Van Slyke and Neill (1924) on p. 540 of their paper. The calculation of  $V_S$  as  $V_S = f_2 P_S$  is an application of the general formula used for reductions of gases to standard conditions; *viz.*,

$$\text{Volume of gas at } 0^\circ, 760 \text{ mm.} = (\text{volume at } t^\circ, P \text{ mm.}) \times \frac{760}{760 (1 + 0.00384t)}$$

In the present case, the volume at  $t^\circ, P$  mm., is  $a$ , the volume of the gas in the chamber when the manometer is read.

The volume per cent of  $CO_2$  in the sample is calculated as:

$$\text{Volume per cent } CO_2 = \frac{100 \times V_{CO_2}}{V_S} = \frac{100 f_1 P_{CO_2}}{f_2 P_S} = \frac{P_{CO_2}}{P_S} \times \text{factor}$$

The factor is  $\frac{100 f_1}{f_2}$ , values of which are given in Table I.

Table II indicates the results obtained by the macro analysis of gas mixtures of varying  $CO_2$  content (from 0.03 to 10 per cent), by the method of isolation described in this section. The results are compared with those obtained by the usual method of Haldane analysis. In the case of Samples 2 and 3 in Table I, these mixtures, because of their small  $CO_2$  content could not be accurately analyzed in the Haldane apparatus. The values given are those calculated by the addition of  $CO_2$ -free air to known, measured volumes of pure  $CO_2$ .

**TABLE II**  
*Determinations of CO<sub>2</sub> in Air by Method A, in Which Both Sample and Isolated CO<sub>2</sub> Are Measured by Pressure*  
 Macro samples measured by pressure at 50 cc. volume

	Sample No.	Manometric analysis		Haldane analysis for CO <sub>2</sub> , 9 to 10 cc. samples
		Approximate size of sample in terms of P <sub>S</sub> at 50 cc.	CO <sub>2</sub> content found	
Air of 0.03 to 1 per cent CO <sub>2</sub> content. P <sub>S</sub> at 50 cc., P <sub>CO<sub>2</sub></sub> at 0.5 cc.	1	150	0.90 0.90	0.88 0.90
	2	520	0.031 0.031 0.031 0.031	0.033*
	3	500	0.079 0.077 0.078 0.080	0.076*
	4	160	3.25 3.30	3.32 3.32
	5	170	5.31 5.25	5.21 5.25
	6	170	10.75 10.61	10.70 10.71
	7	150	3.89 3.92 3.89	3.87 3.86 3.87
	8	140	6.72 6.71 6.78	6.78 6.78 6.78
	9	170	9.94 10.01 9.90 9.93	10.00 9.99
Air of 3 to 10 per cent CO <sub>2</sub> content. P <sub>S</sub> at 50 cc., P <sub>CO<sub>2</sub></sub> at 2 cc.	10	150	3.12 3.13	3.13 3.13
	11	150	3.05 3.05	3.00 3.03
	12	150	10.34 10.34 10.36	10.31 10.32
	13	150	6.98 7.00	6.98 6.97
	14	160	4.91 4.87	4.86 4.88

\* Mixture of known CO<sub>2</sub> content.

Table III covers the results of micro analyses with samples of 0.5 to 1.5 cc.

TABLE III

*Determinations of CO<sub>2</sub> in Air by Method A, in Which Both Sample and Isolated CO<sub>2</sub> Are Measured by Pressure*

Micro samples measured by pressure at 2 cc. volume

	Sample No.	Manometric analysis		Haldane analysis for CO <sub>2</sub> , approximately 10 cc. samples
		Approximate size of sample in terms of $P_S$ at 2 cc.	CO <sub>2</sub> content found	
Air of 17 to 25 per cent CO <sub>2</sub> content. $P_S$ at 2 cc., $P_{CO_2}$ at 0.5 cc.	15	450	25.70 25.65	25.58 25.64 25.69
	16	400	17.62	17.56
			17.65	17.63
				17.64
Air of 3 to 10 per cent CO <sub>2</sub> content. $P_S$ at 2 cc., $P_{CO_2}$ at 0.5 cc.	17	360-510	3.27 3.28 3.34 3.34	3.31 3.35 3.34
			6.63	6.56
			6.54	6.55
			6.60	6.57
	18	370-390	6.67	
			6.69	
			6.52	
			9.43	9.46
	19	360-380	9.38	9.46
			9.36	9.48
			9.41	
			6.44	6.53
	20	370-390	6.46	6.53
			6.45	
			5.89	5.88
	21	370-380	5.92	5.83
			5.91	5.87
			6.50	6.56
	22	430		6.58
			5.86	5.95
	23	500		5.97

*Example of Calculation*—Analysis of CO<sub>2</sub> in air mixture (Sample 14, Table II) gave the following.

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Measured at 23.0° at the 50 cc.	Measured at 23.1° at the 2 cc.
mark	mark
mm.	mm.
$p_1 = 242.2$	$p_2 = 335.7$
$p_0 = \underline{86.0}$	$p_3 = \underline{155.0}$
$P_S = 156.2$	$p_2 - p_3 = 180.7$
	$c = 5.2$
	$P_{CO_2} = 175.5$

Multiplying  $\frac{P_{CO_2}}{P_S}$  by the appropriate factor, 4.330, from Table I, we obtain

$$\text{Volume per cent CO}_2 = \frac{175.5}{156.2} \times 4.330 = 4.87$$

*Corrections for Calibration Errors of Chamber and for Effect of Measuring P<sub>S</sub> Over a Mercury Meniscus*—If  $a$ , the volume at which  $P_S$  is measured, is other than the 50 or 2 cc. assumed in calculating the factors of Table I, the observed  $P_S$  will have to be multiplied by the correction factor  $\frac{a}{2}$  or  $\frac{a}{50}$  to obtain the exact  $P_S$  for use with the factors of Table I. Similarly, if the volume at which  $P_{CO_2}$  is measured is other than the assumed 0.5 or 2.0 cc. the observed  $P_{CO_2}$  will require multiplication by  $\frac{a}{0.5}$  or  $\frac{a}{2}$  in order to obtain the exact  $P_{CO_2}$  for use with the factors of Table I.

In a well calibrated chamber the deviations from the assumed volumes will be negligible, except for  $P_S$  values, measured in the micro determinations, over a mercury meniscus at the 2 cc. mark. The chambers are calibrated for gas measurements over water menisci, and the gas space over a mercury meniscus at a given mark is greater than over a water meniscus at the same mark. If the bore of the chamber at the 2 cc. mark is 4 mm., as is generally the case, we have found that the gas space over a mercury meniscus at that mark is 0.012 cc. greater than over a water meniscus. The value of  $P_S$  measured at a 2 cc. mark exact for a water meniscus will then require multiplication by the correction factor  $\frac{2.012}{2.000} = 1.006$ , when the measurement is made over a mercury meniscus.

In general, if  $a$  represents the supposed gas volume (0.5, 2.0, or 50 cc.) held by the chamber over a water meniscus at a given mark,  $a'$  the actual volume above a water meniscus found in checking the calibration, and  $c_a$  the increase in gas volume measurement that results from changing from a water meniscus to a mercury meniscus, then the observed pressure,  $P_s$ , or  $P_{CO_2}$ , must be corrected by multiplication by  $\frac{a'}{a}$  if the pressure is observed with the gas over an aqueous meniscus, and by  $\frac{a' + c_a}{a}$  when the pressure is observed with the gas over a mercury meniscus. In the  $CO_2$  determinations in gas mixtures above described,  $\frac{a' + c_a}{a}$  is significant only in the measurement of  $P_s$  values at the 2 cc. mark for micro samples. For  $P_s$  measured with the gas at 50 cc. volume, as is the case except when larger samples are taken, the difference between mercury and water menisci is only enough to affect  $P_s$  by about 1 part per 1000, and may ordinarily be neglected.  $P_{CO_2}$  values in this analysis are all measured over water menisci, so that the  $c_a$  correction does not apply to  $P_{CO_2}$ .

#### METHOD B, FOR PRECISE DETERMINATION OF SMALL PROPORTIONS OF $CO_2$ IN AIR

The procedure is the same as the above, except that here a larger sample, 200 or 300 cc. of air, is shaken in successive portions with alkali in the manometric chamber, the absorbed  $CO_2$  being then extracted from solution and measured as above. The 0.03 per cent of  $CO_2$  in ordinary outdoor air can thus be measured with an accuracy of 1 part per 100.

The sample is measured by volume in a container of the type shown on the left in Fig. 1. The container, of, for example, 250 cc. volume, is calibrated by weighing it first empty, except for a film of water on the inner wall, and then with the bulb between the two cocks filled with water. The container, of which the inner walls should be moist, is connected with a mercury leveling bulb, and is completely filled with a sample of the air, the mercury being withdrawn as far as the lower cock. The container is then immersed in a water bath at a temperature about 1° higher than

that of the room, and after thermal equilibrium has been reached, the top stop-cock is exposed to the air and opened for a moment to release the internal pressure. The barometric pressure and the temperature of the water bath are recorded.

The container is then connected to the manometric chamber as shown in Fig. 1 by a flexible rubber tube of about 2 mm. bore and just sufficient length to permit the chamber to be shaken without

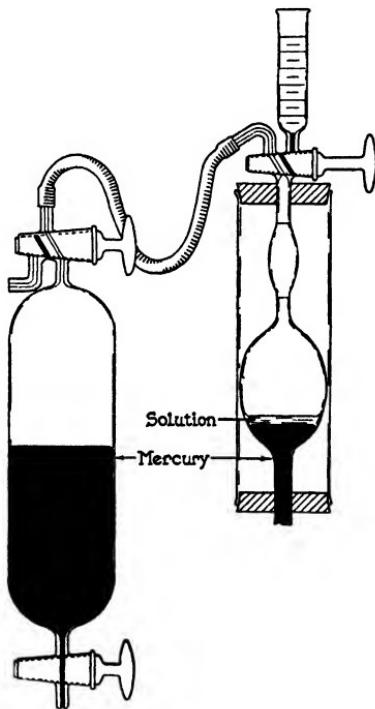


FIG. 1. Method of sampling and analysis of large volumes of gas with the manometric chamber.

disturbing the container. The connecting tube is then filled with mercury from the chamber, and about 35 cc. of the sample are run into the chamber. 3 cc. of the approximately CO<sub>2</sub>-free 0.1 N sodium hydroxide are then admitted to the chamber as above described, and the CO<sub>2</sub> in the air is absorbed by shaking slowly for 4 minutes, with 10 or 15 cc. of mercury in the chamber. The unabsorbed gas, except for a small bubble, is then ejected. Then

another portion of the sample is admitted and its CO<sub>2</sub> is absorbed in the same manner. This procedure is repeated until all the gas from the calibrated container has passed through the chamber and has been shaken with the alkali. When the last portion of gas is run into the chamber a little mercury from the container is permitted to follow and fill the left-hand bore of the cock of the chamber.

After the CO<sub>2</sub> from the last portion of air has been absorbed, the unabsorbed air is ejected without loss of solution, and 0.5 cc. of 1 N hydrochloric acid is admitted to the chamber. The CO<sub>2</sub> is extracted from the solution, and P<sub>CO<sub>2</sub></sub> is measured as directed for Method A. Manometer reading p<sub>1</sub> is taken with the CO<sub>2</sub> gas at 0.5 or 2.0 cc. volume, according to the amount present, and p<sub>2</sub> with the residual gas at the same volume after the CO<sub>2</sub> has been absorbed with 5 N alkali. The value of the c correction is determined in a blank analysis, in which no air is run through the chamber.

### *Calculation*

$$P_{CO_2} = p_1 - p_2 - c$$

$$\text{Volume per cent CO}_2 \text{ in air} = \frac{P_{CO_2} \times 100 f_1}{C \times f_2}$$

C is the capacity in cc. of the container in which the gas sample was measured by volume.

100 f<sub>1</sub> is the factor from Table IV, by which P<sub>CO<sub>2</sub></sub> is multiplied in order to obtain 100 times the cc. of CO<sub>2</sub> present, reduced to 0°, at 760 mm.

f<sub>2</sub> is the usual factor,  $\frac{B - W}{760 (1 + 0.00367t)}$ , by which the observed volume of a moist gas is multiplied to obtain the volume reduced to 0°, 760 mm. B = barometric pressure, corrected for temperature, W = vapor tension of water at the temperature, t° centigrade, at which the gas volume is observed. The values of f<sub>2</sub> are not given here, as they are found in any text-book of gas analysis or of physicochemical tables. In place of values of  $\frac{1}{1 + 0.00367t}$  and values of B corrected for temperature, one may use uncorrected

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values of  $B$  with the factor  $\frac{1}{1 + 0.00384t}$ . The use of the coefficient 0.00384 instead of 0.00367 makes a sufficiently exact correc-

TABLE IV

*Values of Factor, 100 f<sub>1</sub>, by Which P<sub>CO<sub>2</sub></sub> Is Multiplied to Obtain 100 V<sub>CO<sub>2</sub></sub>; for Use in Calculating Results from Method B*

Temperature °C.	Factor when P <sub>CO<sub>2</sub></sub> is measured with gas at 2 cc. volume	Factor when P <sub>CO<sub>2</sub></sub> is measured with gas at 0.5 cc. volume
10	0.2818	0.0718
11	00	14
12	0.2783	09
13	67	05
14	50	01
15	35	0.0697
16	19	93
17	04	89
18	0.2690	86
19	75	82
20	62	78
21	48	75
22	34	71
23	20	68
24	07	65
25	0.2594	61
26	81	58
27	69	55
28	57	52
29	45	49
30	33	46
31	22	43
32	11	40
33	00	37
34	0.2489	34

tion for the expansion of mercury in the barometer, whether the scale is glass or brass (see Van Slyke and Neill, 1924, p. 540).

*Examples of Calculation*—The nature of the results obtained in

analysis of atmospheric air, and of the calculations involved, are indicated by the data in Table V.

TABLE V  
*Calculation of CO<sub>2</sub> Determined in Atmospheric Air by Method B, in Which Sample Is Measured by Volume from Separate Container*  
 Readings of  $p_1$  and  $p_2$  were made with gas at 0.5 cc volume

	Analysis I	Analysis II
Measurement of samples		
$C$ , cc.	291.9	268.6
$t$ , °C	24.3	24.2
$B$ , mm. . .	765.2	765.2
$W$ , "	22.8	22.7
$f_2$	0.897	0.897
Measurement of CO <sub>2</sub> pressures		
Temperature, °C	23.3	21.8
$p_1$ , mm	256.0	270.0
$p_2$ , "	107.3	133.9
$p_1 - p_2$ , mm	148.7	136.1
$c$ , mm	13.6	13.6
$P_{CO_2}$ , mm	135.1	122.5
100 $f_1$	0.0667	0.0672
Calculations		
Volume per cent CO <sub>2</sub>	$\frac{135.1 \times 0.0667}{291.9 \times 0.897} = 0.0344$	$\frac{122.5 \times 0.0672}{268.6 \times 0.897} = 0.0342$

#### SUMMARY

Carbon dioxide is isolated from other gases by shaking the gas mixtures in any desired volume with alkali solution in the chamber of the Van Slyke-Neill manometric apparatus. The other gases are ejected, and the CO<sub>2</sub> absorbed is set free with acid and determined, as in estimations of plasma CO<sub>2</sub> content.

The method is especially adapted to accurate determination of CO<sub>2</sub> when the latter is present in minimal proportions, as in atmospheric air, in which the CO<sub>2</sub> content can be estimated easily within 0.0003 volume per cent. In respired air the method gives results exact to within  $\pm 0.05$  volume per cent.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### III. MANOMETRIC DETERMINATION OF CARBON DIOXIDE TENSION AND pH. OF BLOOD

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Hasselbalch, in 1916, showed that the pH of blood could be calculated by Henderson's mass law equation from the observed  $\text{CO}_2$  tension and the  $\text{CO}_2$  content. Difficulties attending determination of the  $\text{CO}_2$  tension of blood as drawn, however, prevented the immediate application to blood analysis of this simple principle, which promised to obviate the technical difficulties of electrometric methods and the errors of colorimetric ones.

With modern refinements of the technique for  $\text{CO}_2$  determinations in plasma, Eisenman (1926-27) solved the problem by determining the  $\text{CO}_2$  absorption curve of the separated serum (curve with  $\text{CO}_2$  tensions as abscissæ,  $\text{CO}_2$  contents as ordinates), and interpolating on the curve the  $\text{CO}_2$  content of the serum of the shed blood. The abscissa of the curve at the interpolated point indicates the  $\text{CO}_2$  tension.

The present paper offers another procedure, which avoids the necessity of plotting a  $\text{CO}_2$  absorption curve, and of depending upon interpolation. The  $\text{CO}_2$  tension of blood is determined by equilibrating the blood at body temperature with a relatively small bubble of gas, a principle introduced by Pflüger (1872) and applied by Krogh (1908). Under the conditions used, the gas assumes the  $\text{CO}_2$  tension of the blood, which is ascertained by determining the  $\text{CO}_2$  content of the equilibrated bubble. In the present method the bubble is analyzed by the micro gas analysis

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described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932).

A portion of the blood is then centrifuged, and the CO<sub>2</sub> content of the plasma is determined by the Van Slyke-Neill (1924) method. Since conditions of equilibration are such that the CO<sub>2</sub> content of the blood is not significantly changed, the blood used for equilibration can afterwards be used to supply the plasma for analysis. Because of the complications which the cells and the degree of oxygenation introduce by varying the value of pK' in the Henderson-Hasselbalch equation (Warburg, 1922; Van Slyke, Wu, and McLean, 1923), the pH calculation is greatly simplified by basing it on the CO<sub>2</sub> content of plasma or serum rather than on that of whole blood.

From the CO<sub>2</sub> tension and the plasma CO<sub>2</sub> content obtained, the plasma pH is calculated by the Henderson-Hasselbalch equation, with values for pK' and the solubility coefficient of CO<sub>2</sub> determined in previous papers from this laboratory (Hastings, Sendroy, and Van Slyke, 1928; Van Slyke, Sendroy, Hastings, and Neill, 1928). The deviation of the plasma pH values thus gasometrically determined, from values determined with the standard hydrogen electrode, has in none of our analyses exceeded 0.04 pH, and in the majority of analyses has not exceeded 0.02.

The results obtained by the procedure outlined give the acid-base balance of the blood plasma in terms of CO<sub>2</sub> tension, plasma pH, and CO<sub>2</sub> and bicarbonate contents. Sufficient blood remains for oxygen analyses, so that the factors most frequently sought in studies of the acid-base balance are obtained with the one blood portion of 9 cc.

In case conditions render impracticable the anaerobic centrifugation to obtain plasma for CO<sub>2</sub> determination, the CO<sub>2</sub> content and oxygenation may be determined in the whole blood, and from these values and the CO<sub>2</sub> tension the plasma CO<sub>2</sub> may be estimated, with an error not exceeding 2 volumes per cent, by means of the nomogram given in Fig. 2.

One factor of perhaps definite importance is neglected in this, as in all previous methods for routine determination of blood pH. Havard and Kerridge (1929) have reported that shed blood kept at 38° suffers, in a few minutes after drawing, a fall of 0.02 to 0.05 in pH. After this drop they found the pH to remain constant,

except for the slow fall that later sets in as the result of lactic acid formation from glucose. If the blood was cooled to 18° as soon as drawn, the initial pH fall required an hour and a half. These observations, made with glass electrodes, have been confirmed with hydrogen and quinhydrone electrodes by Laug (1930), who found a fall of 0.02 to 0.04 in the pH of plasma when the blood was kept at 36° for 13 minutes after being drawn. There is yet no explanation of the chemical cause of the slight but definite acidification. However, it appears that pH values found in plasma and serum by the techniques commonly applied, and by the one presented in this paper, are about 0.03 lower than the pH in the circulating blood. Such a limited error does not seriously impair the utility of the pH values in acid-base studies.

#### *Error Involved in Equilibration*

If oxygenated blood is equilibrated with air + CO<sub>2</sub>, the CO<sub>2</sub> tension in the gas will approach within 0.3 mm. the original CO<sub>2</sub> tension of the blood, even if the original difference between blood and gas is as much as 10 mm. of CO<sub>2</sub> tension. The degree of approximation is indicated by the following calculation.

If we assume that 1 cc. of gas is equilibrated with 9 cc. of normal blood, and that the initial CO<sub>2</sub> tension of the gas phase is 40 mm., while that of the blood is 50 mm., the gas bubble, in order to raise its CO<sub>2</sub> tension to 50 mm., will take from the blood 0.0126 cc. of CO<sub>2</sub> (calculated at 0°, 760 mm.) and thereby reduce the blood CO<sub>2</sub> content by 0.14 volume per cent. The fall in CO<sub>2</sub> tension caused by removing the 0.0126 cc. of CO<sub>2</sub> from the blood is about 0.3 mm. (see Fig. 91, p. 897, of Peters and Van Slyke, 1931).

The equilibration must be accomplished also without markedly changing the oxygenation of the hemoglobin in the blood, or the CO<sub>2</sub> tension will be altered. Oxygenated hemoglobin acts as a stronger acid than reduced hemoglobin, so that increase in the HbO<sub>2</sub> decomposes BHCO<sub>3</sub> into H<sub>2</sub>CO<sub>3</sub>, with corresponding rise in the CO<sub>2</sub> tension, and *vice versa* (for discussion of this phenomenon, see Peters and Van Slyke, p. 900 *et seq.*, 1931). However, when the gas bubble equilibrated with the blood is only  $\frac{1}{3}$  the volume of the latter, and the initial  $p_{O_2}$  of the blood is set at 80 mm. for arterial, and 25 mm. for venous blood, the changes in oxygenation of the

### 550 III. Manometric Blood CO<sub>2</sub> Tension and pH

hemoglobin are too slight to affect seriously the CO<sub>2</sub> tension.<sup>1</sup> This conclusion may be derived from the following calculation.

9 cc. of normal blood combine with about 1.80 ce. of O<sub>2</sub> to completely oxygenate the hemoglobin. To change the  $p_{O_2}$  of the 1 cc. gas bubble by 10 mm., 0.0126 cc. of O<sub>2</sub> are required, which is enough to saturate 0.007 of the hemoglobin in the 9 cc. of blood. From Fig. 91, p. 897, of Peters and Van Slyke (1931), one can estimate that, with blood CO<sub>2</sub> content constant at 50 volumes per cent, change from complete oxygenation to complete reduction causes a decrease of 13 mm. in the CO<sub>2</sub> tension of blood. Hence, if the HbO<sub>2</sub> of the blood gives to the gas bubble, or the Hb takes from it, enough O<sub>2</sub> to change the  $p_{O_2}$  of the bubble 10 mm., the resultant change in blood CO<sub>2</sub> tension will approximate  $0.007 \times 13$  mm., or about 0.1 mm.

When the initial O<sub>2</sub> tension of the bubble is set at 80 mm. for arterial blood and 25 mm. for venous, it appears that oxygenation changes will rarely affect the CO<sub>2</sub> tension by more than 0.2 mm. (see Fig. 107, p. 987, of Peters and Van Slyke, 1931). In fact, the effect on  $p_{CO_2}$  of measured differences, up to 30 mm., between the initial oxygen tensions of the bubble and the blood has not been experimentally detectable (see Table III).

It appears that equilibration under the conditions used may be depended on to bring the CO<sub>2</sub> tension of the gas bubble within 0.5 mm. of the original CO<sub>2</sub> tension of the blood, and that when greater errors occur in blood  $p_{CO_2}$  determined by the present method they are probably attributable to the micro analysis of the equilibrated gas bubble. This analysis is subject to a maximum error of about 0.2 volume per cent of CO<sub>2</sub>, equivalent to 1.5 mm. of CO<sub>2</sub> tension. The maximum error to be expected in the  $p_{CO_2}$  determination is therefore about  $0.5 + 1.5 = 2$  mm. This is in fact nearly the limit of error we have encountered, as seen in Tables I and II.

#### *Calculation of Plasma pH by Henderson-Hasselbalch Equation, and Sources of Error Involved*

This equation, its derivation from the law of mass action, and its various forms, have been discussed by Austin *et al.* (1922) and

<sup>1</sup> For brevity we shall use the symbols  $p_{CO_2}$  and  $p_{O_2}$  to indicate carbon dioxide tension and oxygen tension, respectively.

on p. 874 *et seq.* of Peters and Van Slyke (1931). The form in which it serves to calculate plasma or serum pH from the CO<sub>2</sub> tension and total CO<sub>2</sub> content of the fluid is expressed in Equations 1 and 2.

$$(1) \quad \text{pH}_s = 6.10 + \log \frac{[\text{CO}_2]_s - 0.067p_{\text{CO}_2}}{0.067p_{\text{CO}_2}}$$

when [CO<sub>2</sub>]<sub>s</sub> is expressed in volumes per cent of CO<sub>2</sub> in the plasma or serum.

$$(2) \quad \text{pH}_s = 6.10 + \log \frac{[\text{CO}_2]_s - 0.0301p_{\text{CO}_2}}{0.0301p_{\text{CO}_2}}$$

when [CO<sub>2</sub>]<sub>s</sub> is expressed in millimols of CO<sub>2</sub> per liter of plasma or serum.

[CO<sub>2</sub>]<sub>s</sub> indicates the total CO<sub>2</sub> content of the serum or plasma,  $p_{\text{CO}_2}$  the CO<sub>2</sub> tension in mm. of mercury, pH<sub>s</sub> the pH of serum or plasma.

These are special forms of the general Henderson-Hasselbalch equation

$$(3) \quad \text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

In Equations 1 and 2 pK' is represented by the constant, 6.10, BHCO<sub>3</sub> is calculated as [total CO<sub>2</sub>] - [H<sub>2</sub>CO<sub>3</sub>], and [H<sub>2</sub>CO<sub>3</sub>] is calculated as

$$(4) \quad \begin{aligned} \text{Volume per cent CO}_2 \text{ as H}_2\text{CO}_3 &= 100 \alpha \times \frac{p_{\text{CO}_2}}{760} \\ &= 0.067p, \text{ when } \alpha = 0.51 \end{aligned}$$

To calculate [H<sub>2</sub>CO<sub>3</sub>] in millimols per liter, the volume per cent factor is multiplied by 10, and divided by 22.26, the volume of 1 mol of CO<sub>2</sub> at 0°, 760 mm. The factor 0.0301 is thus obtained.

It is evident that the precision of the gasometric pH<sub>s</sub> depends upon the accuracy of four values; the [CO<sub>2</sub>]<sub>s</sub> and  $p_{\text{CO}_2}$  determined in the analysis, and the constants, pK' and  $\alpha$ . Each of these four values offers a possible source of error to the calculated pH<sub>s</sub>. It appears that we may estimate these sources of error as follows:

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*Solubility Coefficient of CO<sub>2</sub>*—The value of  $\alpha$  in normal human serum was found by Van Slyke, Sendroy, Hastings, and Neill (1928) to be 0.510, with variations only in the third decimal place. The presence of much lipoid in pathological serum, such as may occur in nephrosis, was found to increase the solubility, sometimes to as high as 0.54, because of the high solubility of CO<sub>2</sub> in fats and oils. However, even in such serum, it is probable that the value 0.510 represents approximately the solubility of CO<sub>2</sub> in the *water phase*, and that the use of the value,  $\alpha = 0.51$ , in calculating [H<sub>2</sub>CO<sub>3</sub>], seldom involves an error of over 1 per cent. An error of 1 part per 100 in [H<sub>2</sub>CO<sub>3</sub>] would alter the calculated pH, by 0.004.

*Total CO<sub>2</sub> Content of Plasma*—In the ordinary 50 cc. form of the Van Slyke-Neill apparatus, plasma CO<sub>2</sub> can be determined with an error not exceeding 1 part in 200. Such an error would affect calculated values of pH, by 0.002. If the plasma CO<sub>2</sub> content is not determined directly, but estimated from blood CO<sub>2</sub> by Fig. 2, the error in [CO<sub>2</sub>]<sub>s</sub> is increased to a possible (though unusual) maximum of 1 part in 20, and the resultant error in calculated pH, is raised to 0.02.

*CO<sub>2</sub> Tension*—When the blood CO<sub>2</sub> tension is determined by the method described in this paper, the maximum error, indicated by Tables I to III, is about 1 part in 20. Such an error in p<sub>CO<sub>2</sub></sub> would cause an error of 0.02 in the calculated pH,.

*The Constant, pK'*—pK' is not an absolute constant, but diminishes as the total electrolyte content of a solution, expressed in terms of ionic strength, increases. The relationship is indicated by the formula  $pK' = 6.33 - 0.5\sqrt{\mu}$  where  $\mu$  represents ionic strength (Hastings and Sendroy, 1925). The increase in electrolyte content caused by adding oxalate to blood should accordingly depress somewhat the pK' value of plasma below that of serum separated without addition of any electrolyte. The data of Cullen (1922), however, indicate a pK' for normal horse oxalated plasma only 0.003 below that for the serum of the same blood. An increase in the value of pK' may be expected in some pathological conditions (especially in severe nephritis) where the total electrolyte content of the plasma is subnormal, so that the use of a normal average pK' for such sera should give pH, results slightly too low. The data of Hastings, Sendroy, and Van Slyke (1928) show, in fact, pK' values in two nephritics higher by 0.02 units than the

average normal value. It is probable that, for serum and plasmas of abnormal electrolyte content, a correction for  $pK'$ , estimated from the deviation of total base from its normal value, could be applied. The magnitude of such correction could be estimated from the formula of Hastings and Sendroy quoted above. Its application to serum and plasma would need to be tested, however, before routine application.

Hastings, Sendroy, and Van Slyke (1928) found in sixteen determinations with human sera, of which six were nephritic, a total range of  $pK'$  values between 6.097 and 6.122 in normal sera, and between 6.108 and 6.134 in nephritic sera. The average of all the values was 6.105, and this agreed with the mean calculated from data of other authors reported since 1922. Accordingly 6.10 was taken as the value of serum  $pK'$ . This value for  $pK'$  has since been generally used, and is employed in calculation of gasometric pH values by the present method. From our data in Table IV,*B*, it can be seen that a  $pK'$  value of 6.11 would give gasometric pH values in slightly closer average agreement with the electrometric values, but the difference is not decisive enough to warrant the slight change from the  $pK'$  value established by the data quoted above.

According to the above considerations a gasometric pH<sub>s</sub> determination by the present method is subject to the following maximum errors from the four values on which the pH<sub>s</sub> calculation depends: 0.002 pH from plasma CO<sub>2</sub> content, directly determined; 0.004 from probable variations in the solubility coefficient,  $\alpha$ , of CO<sub>2</sub> in plasma; 0.010 from variations in  $pK'$ ; 0.020 from errors in determining  $p_{CO_2}$  by the method described in this paper. The total is a maximum error of 0.036 pH<sub>s</sub>, which in fact is about that indicated by the data of Table IV,*B*. The maximum total error is to be expected only in the rare case that all the errors from the four values on which the calculation is based are maximal, and in the same direction.

#### *Apparatus*

The vessel designed for the equilibration of the blood with a prepared gas mixture is shown in *B*, Fig. 1. The body *B* is of 10 cc. total capacity, the smaller bulb being marked for 1 cc. gas volume, leaving 9 cc. for the blood. Stop-cock *S* is bored at a 90°

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angle to allow communication between any two adjacent openings. Stop-cocks and all capillaries leading to them are of 1 mm. bore. The bulb *G*, of 1 cc. capacity, has been introduced so that after blood and gas phase have been brought into equilibrium, the gas may be separated from the blood at 38° before the vessel is removed from the water bath.

#### PROCEDURE

##### *Preparation of Gas Mixtures*

For equilibrating venous blood, a gas mixture having 50 mm.  $p_{CO_2}$  and 25 mm.  $p_O$ , at 38° is used. For arterial blood, the initial gas tensions used are 40 mm.  $p_{CO_2}$  and 80 mm.  $p_O$ . The remainder of the gas mixture may be either nitrogen or hydrogen.

The gas mixtures are stored in 300 cc. containers of the type shown as *T* in Fig. 1. We have prepared the gas mixtures with the aid of the gas manifold described by Austin *et al.* (1922, p. 129). The modification introduced by Van Slyke, Wu, and McLean (1923, p. 805), in which the gas mixtures are made up by pressure measurements, has been used. Gas mixtures may thus be rapidly prepared in which the tensions of CO<sub>2</sub> and O<sub>2</sub> are within 1 mm. of those desired.

*Introduction of Gas into Tonometer*—The larger vessel, *T*, in Fig. 1, contains the prepared gas mixture. *B* and the three capillary tubes at its top are filled with mercury. *G* is filled with mercury from *H*. With cock *S* in position 3, a drop of caprylic alcohol is drawn from cup *C* into the capillary below the cup. *C* is then partially filled with mercury from above, and *B* and *T* are connected as shown in Fig. 1. The connecting capillary *X* is of 0.5 mm. bore, and has at its tip a tapered rubber ring, *R*, shown inserted into cup *C*. About 3 cc. of mercury are admitted into *T* from leveling bulb *A*, then capillary *X* is cleared of air by connecting it with *T* and allowing gas from *T* to waste through *X* and bubble out through the mercury in *C*. The interiors of *B* and *T* are connected by turning the proper cocks, then by lowering the leveling bulb *D* the mercury is withdrawn from *B* and replaced by gas from *T*. Stop-cocks *S'*, *S*, and *F* are closed in the order given. *S* is left closed between positions 2 and 3. Clamp *K* is closed and the rubber tube is disconnected from *S'*. Capillary *X* and the mercury are removed from cup *C*.

*Drawing Blood and Introducing It into Tonometer*—The blood is drawn into a tube coated with enough dried neutral potassium oxalate and ammonium fluoride to make the final concentrations in the blood 0.2 and 0.1 per cent, respectively. The necessary volume of a *neutral* solution containing 20 gm. of potassium oxalate

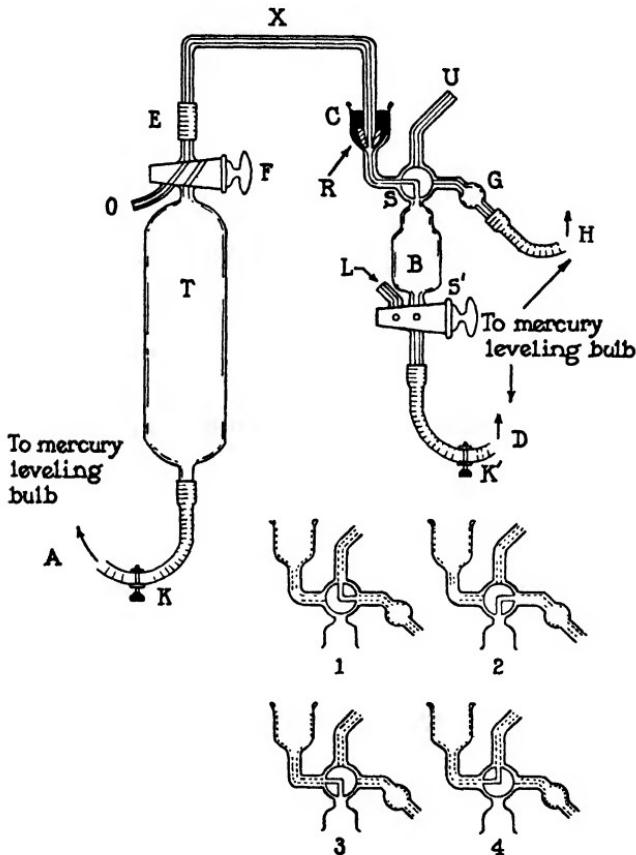


FIG. 1. Apparatus for equilibration of blood. *B* is the tonometer in which the blood is to be equilibrated. *T* is a gas container, from which *B* is about to be filled with a gas mixture approximating the CO<sub>2</sub> and O<sub>2</sub> tensions of ordinary venous or arterial blood.

and 10 gm. of ammonium fluoride per 100 cc. is spread on the inside of the vessel and dried with a current of air. The blood is drawn without contact with air. For this purpose it may be

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drawn under oil, as described by Van Slyke and Cullen (1917). However, it is preferable to draw it directly into a closed tube over mercury, as described on p. 131 of Austin *et al.* (1922). If first drawn into a tube with oil, the blood is transferred to a closed tube over mercury (tube *J*, Fig. 3, of Austin *et al.*, 1922).

The tonometer, *B*, previously filled with the desired gas mixture, is connected to the blood tube through the lower cock, *S'*. A few drops of blood are run through the outlet *L*. Then stop-cock *S* is opened in position 3 to let out displaced gas, and the vessel is filled through *S'* with blood to the 1 cc. mark. Cock *S* is closed in position midway between positions 3 and 4. Leveling bulb *D* is again connected to the lower stem of tonometer *B*, and the stem is cleared of blood by mercury from *D* wasted through the outlet *L*.

As an alternative procedure, the blood may be drawn into a syringe containing paraffin oil, and forced directly from the syringe into tonometer *B* through a short rubber tube of 2 mm. bore. In this case the necessary layer of oxalate and fluoride is placed on the inner wall of the syringe. During the delivery of blood from the syringe into *B*, the point of the syringe is held downwards, with the connecting tube to *B* bent into a U, so that no oil may enter *B*.

*Equilibration of Blood and Gas at 38°*—The tonometer *B* in an upright position is immersed as far as the upper cock in water at  $38^\circ \pm 0.1^\circ$ . Leveling bulbs *D* and *H*, attached to the tonometer, are suspended outside the bath. A droplet of mercury is placed in cup *C*. The tonometer is held in the bath for 1 or 2 minutes, then *S* is turned to position 3 to allow escape of enough of the warmed air to lower the pressure within the chamber to atmospheric. The escape of gas is indicated by movement of the droplet of mercury in cup *C*. Cock *S* is closed and the tonometer is left in the bath for another minute, after which *S* is again opened. This procedure is repeated until there is no further indication of the escape of gas when the cock is opened.

Cock *S* is turned from position 3 in a clockwise direction to a position midway between positions 1 and 2. A rubber stopper is inserted into the mouth of cup *C* to keep out water from the bath. The entire tonometer is then immersed in the bath and is rocked in such a manner that the bubble moves from one end of the chamber to the other. An automobile wind-shield wiper, run by

compressed air or vacuum may be used for this purpose. 10 minutes suffice for attainment of CO<sub>2</sub> equilibrium. At the end of that period the tonometer, still in the bath, is placed in an upright position for 1 or 2 minutes to permit drainage of blood from the wall of the upper part of the chamber. Then, with leveling bulb *H* slightly elevated, stop-cock *S* is turned to position 2 just long enough to permit a droplet of mercury from *G* to pass into the chamber *B*. The mercury removes blood from the bore of cock *S*, from which it might otherwise enter *G* when the gas is transferred to this bulb.

*Separation of Equilibrated Blood and Gas*—This operation is preferably performed without removing the tonometer from the bath. If one works quickly, however, the tonometer may be taken out and the gas bubble transferred to bulb *G* before temperature change has significantly affected the distribution of CO<sub>2</sub> between the gas and blood.

The tonometer is either removed from the bath, or, preferably, placed in an upright position with only the part above cock *S* above the surface of the bath. The stopper is removed from *C*. Leveling bulb *H* is placed slightly below and leveling bulb *D* slightly above the tonometer. Cock *S* is then turned to position 2. A portion of the gas from *B* escapes at once into *G*. Most of the remaining gas is driven into *G* by admitting mercury from leveling bulb *D* into the bottom of chamber *B*. The admission of mercury is stopped when almost all of the gas has been transferred to *G*, and before any blood has entered the bore of cock *S*. Cock *S* is then turned to position 3 and the small bubble of gas left in *B*, followed by a little blood, is allowed to escape into cup *C*.

*S* is turned to position 4, and cup *C* and the bore of the cock are cleaned by drawing water, and then acetone in succession through *U*. The separated gas and blood may now be analyzed at the operator's convenience. If, however, the blood is not analyzed at once, it should be chilled in ice water and kept cold until used. Even then the blood analysis should be made on the same day. Before removal of either the blood or gas for analysis, the tonometer should be brought to room temperature.

*Determination of CO<sub>2</sub> Content of Gas Bubble*—This analysis is carried out as described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932). The technique described for measuring micro gas samples is followed. To transfer the gas sample from

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bulb *G* of Fig. 1 to the Van Slyke-Neill manometric chamber, the arm *U* is connected glass to glass with the side arm of the chamber. Mercury is then run back and forth between cup *C* of the tonometer and the Van Slyke-Neill chamber to drive all gas bubbles out of the connections. Manometer reading  $p_0$  is taken, with the meniscus of the mercury at the 2 cc. mark in the gas-free manometric chamber. The mercury leveling bulb attached to *H* is then placed higher than the leveling bulb of the manometric apparatus, cock *S* is turned to position 1, and all the gas in *G* is passed into the manometric chamber followed by a little mercury to seal the cock of the chamber. The mercury meniscus in the chamber is again brought to the 2 cc. mark and manometer reading  $p_1$  is taken. The pressure exerted by the gas sample at 2 cc. volume is calculated as

$$P_S = p_1 - p_0$$

The absorption of CO<sub>2</sub> with NaOH solution and the rest of the analysis are then carried out as described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932).

*Centrifugation of Blood and Determination of Plasma CO<sub>2</sub> Content*—Tube *X* of Fig. 1 is replaced by another glass capillary, of which the descending outlet limb is long enough to reach to the bottom of a centrifuge tube. The blood is then passed into a centrifuge tube containing a layer of oil. The oil is at once replaced by a layer of low melting paraffin, and the blood is centrifuged. The paraffin is pierced with a warm cork-borer, and 1 cc. samples of the plasma are withdrawn into pipettes and used for determination of the CO<sub>2</sub> content of the blood, according to Van Slyke and Neill (1924).

#### *Calculation*

From the volume per cent CO<sub>2</sub> content of the gas bubble, *C*, the CO<sub>2</sub> tension is calculated by the usual formula.

$$p_{CO_2} = 0.01 C (B - 49)$$

where *B* is the barometric pressure in mm. of mercury and 49 is the vapor tension of water at 38°.

From the value of  $p_{CO_2}$  and the CO<sub>2</sub> content of the plasma, the plasma pH is calculated by Equation 1 or 2, previously given. Or

the calculation may be made graphically by the line-chart given in Fig. 1 of Van Slyke and Sendroy (1928), and reproduced in Fig. 87 of Peters and Van Slyke (1931).

*Estimation of Plasma CO<sub>2</sub> Content from Whole Blood CO<sub>2</sub> Content and CO<sub>2</sub> Tension*—The chart in Fig. 2 is analogous to that in Fig.

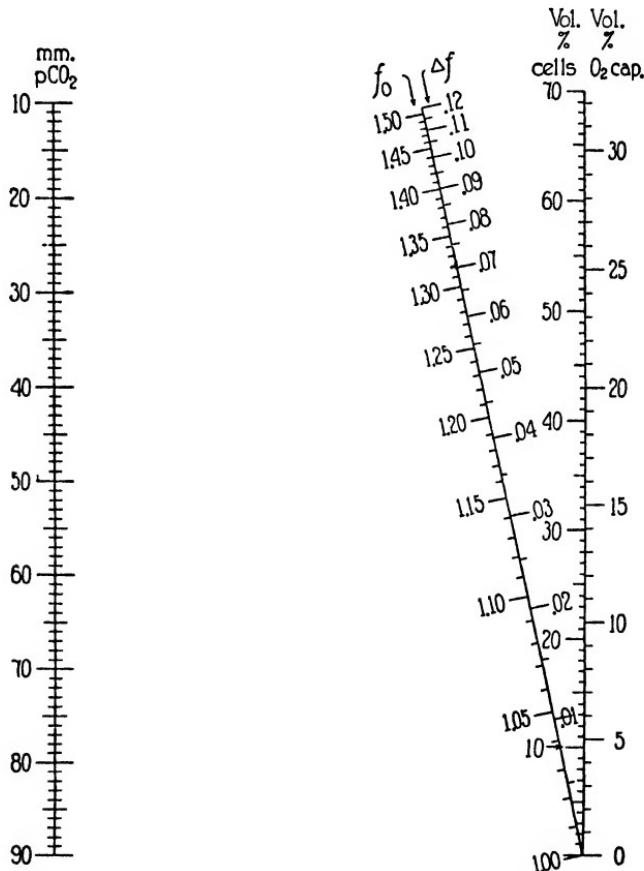


FIG. 2. Line-chart for estimating plasma CO<sub>2</sub> content from whole blood CO<sub>2</sub> content.

3 of Van Slyke and Sendroy (1928) for estimating the CO<sub>2</sub> content of plasma from that of whole blood, with the aid of a factor dependent on the oxygen capacity and pH<sub>s</sub> of the blood. In the present chart, Fig. 2, a scale for pCO<sub>2</sub> replaces that for pH<sub>s</sub>. The

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use of the chart is exactly the same as that of the former one, except that  $p_{CO_2}$  values are used in place of pH<sub>s</sub>. The CO<sub>2</sub> tension values are more convenient in connection with the determinations outlined above, because the  $p_{CO_2}$  is directly determined. Theoretically, the use of  $p_{CO_2}$  is not so precise as the use of pH<sub>s</sub> to correct for the effect of reaction changes on the distribution of bicarbonate, in accordance with Donnan's law, between cells and plasma. In practice, however, we have found little difference between the two charts in the accuracy with which they estimate plasma CO<sub>2</sub> content from whole blood values. The error in plasma CO<sub>2</sub> estimation is usually less than 1 volume per cent, but may be as great as 2.5 volumes per cent.

Fig. 2 can be used to estimate plasma CO<sub>2</sub> contents from whole blood values when working conditions are not convenient for centrifuging the blood to obtain plasma for direct analysis. Any such indirect estimation of plasma CO<sub>2</sub> content, however, increases by about 0.02 the possible error in the pH<sub>s</sub> calculated, as has been already shown in the discussion of "Calculation of plasma pH by Henderson-Hasselbalch equation, and sources of error involved."

#### EXPERIMENTAL

##### *Blood CO<sub>2</sub> Tensions Set by Saturation with Known Gas Mixtures Compared with Tensions Subsequently Found in Blood by Present Method*

Blood was treated with oxalate and fluoride, as directed for the present method. Then, in order to set the CO<sub>2</sub> and O<sub>2</sub> tensions of the blood at known levels, it was subjected to preliminary saturation with large volumes of gas, in which CO<sub>2</sub> was mixed with H<sub>2</sub>, or with H<sub>2</sub> and O<sub>2</sub>, or with air. The "first saturation method" of Austin *et al.* (1922) was used, in which a tonometer with two chambers is employed, such that, after saturation is finished, the gas phase can be separated for analysis in a large chamber and the blood in a small one. The composition of the gas, with which the blood had been saturated, was determined in a Haldane air analysis apparatus. From the analysis of the gas the CO<sub>2</sub> tension of blood present, given in Tables I to III, was calculated, and, in Table III, also the O<sub>2</sub> tension of blood present. The limit of error of the Haldane air analysis is 0.03 volume per cent of CO<sub>2</sub> or O<sub>2</sub>,

corresponding to 0.2 mm. tension of these gases. The CO<sub>2</sub> tension of blood present may therefore be considered to be ascertained within 0.2 mm.

Of the blood thus prepared, 9 cc. portions were transferred to tonometer *B* of Fig. 1, and were equilibrated as described in this paper, with 1 cc. portions of analyzed gas mixtures. A 1 cc. bubble was used, consisting of CO<sub>2</sub> and H<sub>2</sub> for previously reduced blood

TABLE I

*Determinations of CO<sub>2</sub> Tension in Reduced Horse and Ox Blood Previously Saturated with Hydrogen Gas Containing Known Tensions of CO<sub>2</sub>*

Blood No.	Initial CO <sub>2</sub> tension of gas bubble	CO <sub>2</sub> tension of blood		
		Present	Found from final pCO <sub>2</sub> of gas bubble	Error
	mm.	mm.	mm.	mm.
1	45.8	41.8	42.5	+0.7
2	45.8	51.6	49.3	-2.3
3	44.1	50.7	51.8	+1.1
4	42.6	38.0	36.3	-1.7
5	42.6	50.2	47.9	-2.3
6	36.8	37.2	37.0	-0.2
7	36.8	44.9	44.3	-0.6
8	44.4	45.6	46.9	+1.3
9	44.4	38.1	38.7	+0.6
10	45.3	50.6	53.0	+2.4
11	45.3	40.8	39.0	-1.8
12	44.7	49.2	48.3	-0.9
13	44.7	40.5	41.5	+1.0
14	45.5	48.6	47.2	-1.4
15	45.5	40.1	41.9	+1.8
16	42.5	35.4	37.1	+1.7
17	42.5	47.6	46.9	-0.7

(Table I), of CO<sub>2</sub> and air for previously oxygenated blood (Table II), and of CO<sub>2</sub>, H<sub>2</sub>, and O<sub>2</sub> for blood oxygenated in varying degrees (Table III). The 1 cc. portions of gas used were prepared with CO<sub>2</sub> tensions, and, in Table III, O<sub>2</sub> tensions, differing to varying extents from the previously set tensions of the blood, but within such limits of difference as are likely to occur in determinations of the CO<sub>2</sub> tension of drawn venous or arterial blood.

The results indicate that the error of blood CO<sub>2</sub> tensions de-

TABLE II

*Determinations of CO<sub>2</sub> Tension in Oxygenated Ox Blood Previously Saturated with Air Containing Known Tensions of CO<sub>2</sub>*

Blood No.	Initial CO <sub>2</sub> tension of gas bubble	CO <sub>2</sub> tension of blood		
		Present	Found from final pCO <sub>2</sub> of gas bubble	Error
1	mm. 37.6	mm. 44.7	mm. 43.6	mm. -1.1
2	47.8	43.7	43.0	-0.7
3	44.1	41.6	43.1	+1.5
4	34.2	41.6	41.6	0.0
5	38.4	43.3	43.3	0.0
6	38.4	45.0	45.0	0.0
7	38.4	46.1	44.7	-1.4
8	38.4	36.0	38.3	+2.3
9	43.0	45.9	46.7	+0.8
10	43.0	36.3	36.9	+0.6
11	40.7	45.9	46.5	+0.6
12	40.7	36.3	35.5	-0.8
13	43.6	37.0	38.7	+1.7
14	45.7	37.0	37.7	+0.7
15	40.8	37.0	39.4	+2.4
16	41.7	44.4	44.7	+0.3
17	39.4	44.4	42.4	-2.0
18	43.5	49.1	49.8	+0.7
19	38.5	49.1	50.0	+0.9

TABLE III

*Determinations of CO<sub>2</sub> Tension in Ox, Horse, and Human Blood of Varying Degrees of Oxygenation*

Each blood was previously saturated with a gas mixture containing CO<sub>2</sub>, H<sub>2</sub>, and O<sub>2</sub> at known tensions.

Blood No.	Initial tensions of gas bubble		Gas tensions present in blood		CO <sub>2</sub> tension found in blood	Error in CO <sub>2</sub> tension found
	CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>		
1	mm. 41.2	mm. 140.3	mm. 46.6	mm. 139.2	mm. 46.7	mm. +0.1
2	43.5	52.3	46.6	139.2	43.1	-3.5
3	43.7	73.8	52.3	54.7	51.8	-0.5
4	43.7	73.8	39.9	102.1	39.4	-0.5
5	42.3	68.0	43.9	86.8	45.0	+1.1
6	42.3	68.0	44.3	50.0	43.4	-0.9
7	41.2	66.8	38.9	85.4	38.6	-0.3
8	41.2	66.8	46.8	47.9	46.2	-0.6
9	51.7	30.3	57.5	47.8	57.3	-0.2
10	51.6	41.4	57.5	47.8	59.6	+2.1
11	46.5	61.5	57.5	47.8	59.4	+1.9
12	45.6	70.9	52.8	47.1	51.2	-1.6

termined as described in this paper averages approximately 1 mm. Except for Blood 2 of Table III, in which the initial oxygen tension of the equilibrating bubble was intentionally made greatly different from that of the blood, the maximum error is 2.4 mm. of CO<sub>2</sub> tension.

*Comparison of Electrometric and Gasometric pH. Determinations in Human Venous Blood*

The blood was obtained partly from hospital patients and partly from normal individuals. It was collected over mercury without contact with air, as described by Austin *et al.* (1922), in tubes provided with potassium oxalate and fluoride to prevent coagulation and lactic acid formation. No particular effort was made to prevent stasis, hence the CO<sub>2</sub> tensions are a little higher and the pH. values a little lower than usual for blood drawn from the arm vein without stasis. Each sample of blood was divided into two portions.

Portion I was transferred to tonometer *B* of Fig. 1, and was used for determination of the CO<sub>2</sub> tension by the method described in this paper. After the gas bubble had been withdrawn from the tonometer for analysis, the residual blood in *B* was used for determinations of blood CO<sub>2</sub> content, oxygen content, and oxygen capacity by the methods of Van Slyke and Neill (1924). The results of the analyses are given in Table IV, *A*.

Portion II was transferred to a centrifuge tube containing a layer of paraffin oil, which was at once replaced by melted paraffin. The blood was centrifuged under the solidified paraffin. The separated plasma was drawn into a tube over mercury, as described by Austin *et al.* (1922), and was used for electrometric determination of the pH, and, in Bloods 9 to 13, for determination of the plasma CO<sub>2</sub> content.

For the electrometric pH determination at 38° the electrode chamber of Clark (1915) was used, with thermometer added as described by Cullen (1922). The electrode was filled with about equal volumes of plasma and gas. The gas used was hydrogen to which sufficient CO<sub>2</sub> was added to give in each case the pCO<sub>2</sub> which the blood was found to have by the writers' method in Portion I.

The H<sub>2</sub>-CO<sub>2</sub> gas mixtures used in the electrometric pH. deter-

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TABLE IV  
A. Analyses of Venous Human Blood as Drawn

Blood No.	O <sub>2</sub> capacity of blood	O <sub>2</sub> saturation of blood	CO <sub>2</sub> content of whole blood = [CO <sub>2</sub> ] <sub>b</sub>	CO <sub>2</sub> content of plasma = [CO <sub>2</sub> ] <sub>p</sub>		Determinations of CO <sub>2</sub> tension		
				Determined directly on separated plasma	Estimated from [CO <sub>2</sub> ] <sub>b</sub> by Fig. 2	Initial tensions in gas bubble used in tonometer	Final pCO <sub>2</sub> in gas bubble = blood pCO <sub>2</sub>	
	vols. per cent	per cent	vols. per cent	vols. per cent	vols. per cent	CO <sub>2</sub>	O <sub>2</sub>	mm.
1	20.93	77.3	57.88	69.27	45.2	67.7	60.4	
			57.91	69.72	58.3	67.1	59.4	
2	21.55	58.0	55.74	67.05	45.2	67.7	55.1	
			55.52	66.65	58.3	67.1	56.5	
3	21.48	61.0	53.61	64.10	48.3	67.1	57.2	
			53.87	64.70	45.2	67.7	53.6	
4	18.46	69.0	55.18	64.98	60.0	44.6	50.2	
			55.16	64.95	45.8	67.8	50.0	
5	19.77	61.8	54.03	63.93	60.0	44.6	52.0	
6	12.47	39.0	61.42	67.45	60.0	44.6	61.8	
			61.53	67.60	45.8	67.8	61.9	
7	19.78	60.1	57.70	67.92	60.0	44.6	60.2	
			58.01	68.30	45.8	67.8	60.7	
8	10.68	41.7	60.55	65.52	60.0	44.6	60.9	
9	20.77	84.2	54.14	65.62	65.76	44.8	40.2	48.9
10	16.41	55.4	55.08	62.49	62.77	51.6	41.4	55.0
11	21.04	72.3	52.93	63.22	63.78	51.6	41.4	49.5
12	22.42	61.6	53.80	65.04	65.18	46.5	61.5	54.5
13	19.86	65.2	48.00	57.03	56.23	51.7	30.3	57.3
			48.30	57.03	56.50	51.6	41.4	59.6
			48.09	57.03	56.38	46.5	61.5	59.4
14	21.98	42.0	62.00	74.06	46.3	23.7	65.3	
			62.00	73.90	49.1	13.8	65.8	
15	20.23	67.0	57.14	68.11	46.3	23.7	54.0	
			57.14	67.96	47.5	33.2	55.3	
16	23.51	80.1	48.82	60.60	40.3	23.4	47.3	
			48.93	60.58	48.7	14.7	47.7	
			48.87	60.60	48.4	0.0	47.5	
17	19.95	50.8	56.76	66.80	46.3	23.4	56.6	
			57.17	67.33	48.7	14.7	56.3	
			57.19	67.27	48.4	0.0	58.6	
18	21.25	31.4	59.17	69.74	46.0	23.5	65.3	
			59.36	69.97	48.6	0.0	65.4	

TABLE IV—Concluded  
B.  $pH_s$  Values Found in Bloods of Table IV, A

Blood No.	Gasometric $pH_s$		$pH_s$ by $H_2$ electrode	Error of gasometric $pH_s$ if electrometric is exact	
	From $pCO_2$ and directly determined $[CO_2]_s$	From $pCO_2$ and $[CO_2]_s$ estimated from whole blood $CO_2$		Gasometric $pH_s$ from direct $[CO_2]_s$	Gasometric $pH_s$ from $[CO_2]_s$ esti- mated from $[CO_2]_b$
1		7 30	7 32		-0 02
		7 32	7 32		±0 02
2		7 33	7 35		-0 02
		7 32	7 35		-0 03
3		7 30	7 30		0 00
		7 33	7 30		+0 03
4		7 36	7 39		-0 03
		7 36	7 39		-0 03
5		7 34	7 38		-0 04
6		7 28	7 31		-0 03
		7 28	7 31		-0 03
7		7 30	7 32		-0 02
		7 30	7 32		-0 02
8		7 27	7 29		-0 02
9	7 37	7 38	7 39	-0 02	-0 01
10	7 31	7 30	7 33	-0 02	-0 03
11	7 36	7 36	7 40	-0 04	-0 04
12	7 33	7 33	7 33	±0 00	0 00
13	7 24	7 23	7 20	+0 04	+0 03
	7 22	7 22	7 20	+0 02	+0 02
	7 22	7 22	7 20	+0 02	+0 02
14		7 30	7 32		-0 02
		7 30	7 32		-0 02
15		7 34	7 34		0 00
		7 34	7 34		0 00
16		7 31	7 36		0 00
		7 35	7 36		-0 01
		7 36	7 36		0 00
17		7 32	7 31		+0 01
		7 33	7 31		+0 02
		7 30	7 31		-0 01
18		7 27	7 27		0 00
		7 27	7 27		0 00

### 566 III. Manometric Blood CO<sub>2</sub> Tension and pH

minations were made up by pressure as described above for "Preparation of gas mixtures." In this case, however, where exactness was necessary, a correction was required for the change in vapor tension of water between the room temperature, at which the gas mixture was made, and the electrode temperature of 38° to which the plasma and gas were brought for the pH determination. If one represents barometric pressure by  $B$ , room temperature by  $t$ , vapor tension of water at 38° by  $W_{38}$ , and vapor tension of water at  $t^{\circ}$  by  $W_t$ , the CO<sub>2</sub> pressure that must be measured at room temperature is calculated as follows:

$$\text{Measured } p_{\text{CO}_2} \text{ at } t^{\circ} = \text{desired } p_{\text{CO}_2} \text{ at } 38^{\circ} \times \frac{B - W_t}{B - W_{38}}$$

Mixtures made by this method and then subjected to gas analysis were found to be within 1 mm. of the desired CO<sub>2</sub> tension, and usually within 0.5 mm. Any difference in CO<sub>2</sub> tension between plasma and gas put into the electrode chamber would be reduced to less than half by interchange between plasma and gas, so that the final CO<sub>2</sub> tension in the plasma used for electrometric pH estimation can be assumed to be within less than 0.5 mm. of the tension found by our method in blood Portion I. An error of 0.5 mm. in setting the CO<sub>2</sub> tension in the electrode chamber would affect the determined pH by approximately 0.005. It appears probable that the total error of the electrometric pH determination may be considered to be within the limit of  $\pm 0.01$  pH.

The electrodes were standardized with 0.1 N hydrochloric acid, which was assumed to have a pH of 1.08. The system used and the standardization have been discussed, on pp. 708-709 of a previous paper (Van Slyke, Hastings, Murray, and Sendroy, 1925).

The results in Table IV,  $B$  show a maximum deviation of the gasometric pH, from the electrometric of  $\pm 0.04$  pH. In the majority of cases the deviation does not exceed 0.02 pH.

Since the observed deviations represent the sum of errors between the gasometric and electrometric determinations, it appears that the maximum error in the gasometric pH, does not usually exceed 0.03 pH.

#### SUMMARY

Gasometric methods are described for determining in blood the carbon dioxide tension and the plasma pH.

The CO<sub>2</sub> tension is obtained by equilibrating blood with  $\frac{1}{5}$  its volume of a gas mixture which contains CO<sub>2</sub> and O<sub>2</sub> in tensions approximating those of average venous or arterial blood. The gas bubble attains the CO<sub>2</sub> tension of the blood, which is then determined by micro gas analysis of the bubble with the method described in the preceding paper.

The pH of the plasma is calculated by the Henderson-Hasselbalch equation from the CO<sub>2</sub> tension found in the blood and the CO<sub>2</sub> content determined by analysis of subsequently separated plasma.

The maximum errors are  $\pm 2.5$  mm. of CO<sub>2</sub> tension and  $\pm 0.04$  pH; the usual errors are less.

With one sample of blood and an entirely gasometric technique carried out with the manometric apparatus, these determinations give the acid-base balance of the plasma in terms of pH, CO<sub>2</sub> or bicarbonate content, and CO<sub>2</sub> tension.

The writers are much indebted to Dr. A. Alving and Dr. J. M. Steele for obtaining samples of human blood.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

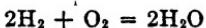
### IV. HYDROGEN AND OXYGEN BY COMBUSTION

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The method here described is an adaptation to the Van Slyke-Neill (1924) manometric apparatus of the familiar principle of hydrogen determination by ignition. The gas is ignited with excess oxygen and the hydrogen is calculated as  $\frac{2}{3}$  of the gas which disappears, in accordance with the reaction:



The method is, of course, applicable without modification only when other combustible gases are absent. An analysis requires about 20 minutes.

For gas analysis by combustion the manometric apparatus, compared with ordinary gas burettes, offers two advantages. (1) Accurate results can be obtained with samples of widely varying size. As little as 1 cc. suffices for analysis with 0.2 volume per cent accuracy, or as much as 30 cc. can be taken, whereby the error is reduced to about 0.05 volume per cent of hydrogen. (2) When the gas mixture is explosive (over 10 per cent hydrogen), it is not necessary to dilute it with air to reduce the explosibility. This object is attained merely by reducing the pressure on the gas.

The same technique serves for oxygen determination when an excess of hydrogen is present. In this case, since combustion diminishes the gas present by 3-fold the amount of oxygen, the analysis can be made with a maximum error of only 0.02 or 0.03 volume per cent of  $\text{O}_2$ .

The general principles of technique and precautions for mano-

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metric gas analyses outlined in the introduction to Paper I of this series (Van Slyke and Sendroy, 1932) are to be observed.

### Apparatus

Fig. 1 illustrates the construction and manner of attachment of the combustion chamber to the reaction chamber of Van Slyke and Neill. The combustion chamber consists of a heavy walled Pyrex glass tube, of about 100 mm. length and 32 mm. diameter, and therefore about 75 cc. capacity. The mouth of the cylinder

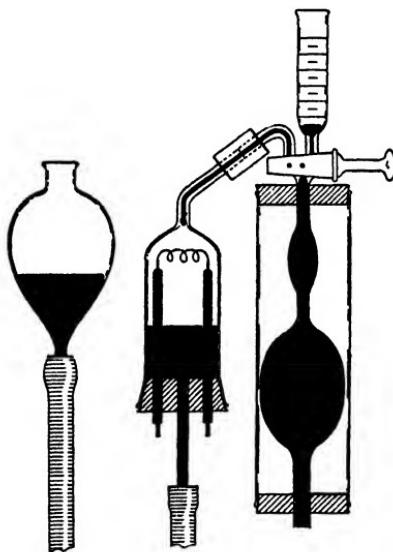


FIG. 1. Manometric and combustion chambers connected. The leveling bulb shown is connected with the combustion chamber.

is expanded slightly so that it will fit the rubber stopper. The glass here is also thickened somewhat and a rim is put on the edge, so that the greased stopper can be forced in without breaking the glass.

Through the stopper pass three glass tubes. One is connected by about 100 cm. of rubber tubing with the mercury leveling bulb. Into the upper ends of the other two are sealed short heavy platinum wires, which are joined within the chamber by a thin platinum wire (No. 26 or 28) about 5 cm. long, bent into three spirals.

After the platinum wires are sealed into the glass, each tube is filled with melted Wood's metal, and a strong copper wire is inserted into the cooling metal. This device, suggested by Dr. Sendroy, enables one to economize on platinum, and to use strong and heavy copper wires for connections below the tubes with the battery wires. Electricity to heat the platinum spiral is provided by a battery of two or three dry cells.

To avoid danger from flying glass in case a miscalculation of the mixture burned should cause a highly explosive one to be ignited (an accident which has not yet occurred), it is well to wrap a layer of wire gauze about the combustion chamber, or slip a mica lamp chimney about it.

The ends of the capillaries connecting the combustion and manometric chambers are ground flat so that they meet with a minimum of dead space. The rubber tube connecting the capillaries from the two chambers is new, heavy walled red "nitrometer" tubing with a small enough bore so that it grips the glass tubes firmly. The same sort of tubing is used to connect the combustion chamber with its leveling bulb.

*In order to insure joints which will not leak under reduced pressure* both the rubber tubing and the rubber stopper used on the combustion chamber are cleaned and boiled with dilute alkali before use, and where rubber fits against glass both surfaces are covered with thin layers of grease before they are joined. The stopper is held tightly in place by wires. It is unnecessary to bind with wire or otherwise the short rubber tube connecting the capillaries of the two chambers. Binding is undesirable because it makes the rubber tube spread slightly, and thereby increases the dead space between the ends of the glass capillaries. The combustion chamber is supported at such a level that its capillary meets that of the Van Slyke-Neill chamber exactly. With these precautions we have never had any perceptible leaks when the pressure in the combustion chamber was reduced to 100 mm. (leveling bulb lowered 660 mm.), which is the maximum evacuation used.

If the leveling bulb of the combustion chamber is lowered so far that a complete Torricellian vacuum is obtained in this chamber, a slight amount of air may enter by diffusion through the thick rubber tube joining the connecting capillaries of the two chambers. To avoid obtaining such a vacuum when evacuating

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the combustion chamber, we hang the leveling bulb by a hook from the links of a chain, the lowest link of which does not permit the surface of the mercury in the bulb to fall by more than 660 mm. below the floor of the combustion chamber. (The type of chain used for supporting window sash is convenient.)

The combustion chamber can be easily attached or detached in 1 minute, so that a combustion can form one step in a series of analyses, of which the others are made without the combustion chamber.

In some Van Slyke-Neill manometric chambers the curved outlet capillary approaches so near to the end of the cock that either the bend of the capillary must be changed or the end of the cock ground away to make room for the heavy rubber tube used to attach the combustion chamber.

### *Reagents*

The only reagents required are oxygen and hydrogen, an excess of either being used in determination of the other.

Oxygen can be used in the form of room air for determination of hydrogen, but causes an undesirably great dilution of the sample if the hydrogen content of the latter exceeds 10 per cent. For gas mixtures with higher hydrogen contents pure oxygen is used as the reagent.

Either oxygen or hydrogen can be conveniently introduced into the manometric chamber from a modified Hempel pipette, in the manner illustrated on p. 809 of the paper by Van Slyke and Hiller (1928).

### DETERMINATION OF HYDROGEN IN LARGE SAMPLES, BY PRESSURE MEASUREMENTS WITH THE GAS AT 50 CC. VOLUME

#### *Preparation of Apparatus to Receive Sample*

The combustion chamber is attached and its leveling bulb is routinely located as shown in Fig. 1.

Before the gas sample is admitted a Torricellian vacuum is created in the manometric chamber. Then the connection with the combustion chamber is opened for a second or two. Any air in the connecting capillaries is swept by the rush of mercury into the manometric chamber, from which it is then ejected.

The zero reading,  $p_0$ , is taken with the meniscus of the mercury at the 50 cc. mark, as described by Van Slyke and Sendroy (1932). The gas sample is then admitted as described by the same authors for "Admission of sample estimated by volume," and the  $p_1$  reading is taken as described by them for "Measurement of sample" (p. 518). The pressure  $P_s$  of the sample at 50 cc. volume is measured as  $P_s = p_1 - p_0$ .

#### *Transfer of Sample to Combustion Chamber*

After  $p_1$  has been observed all the gas sample is passed over into the combustion chamber. For the transfer, the leveling bulb of the combustion chamber is lowered a little below the level of the chambers, while the leveling bulb of the manometric chamber is left level with the chambers. The cock at the top of the manometric chamber is opened to connect the two chambers. Mercury is admitted into the bottom of the manometric chamber until all the gas, followed by a little mercury, has been driven over into the combustion chamber. The leveling bulb of the manometric chamber is raised only as much as is necessary to permit the last portions of gas to pass into the combustion chamber. The transfer of gas is accomplished without at any time putting the gas in the manometric chamber under positive pressure, which would be undesirable, since it might cause leakage of some gas out of the cock at the top of the chamber. (The cock is designed to hold against a complete internal vacuum, but cannot be trusted always to hold against positive pressure inside the chamber.)

#### *Addition of Air or Oxygen*

After the sample has been transferred to the combustion chamber enough air or oxygen to burn the hydrogen is measured into the manometric chamber. The desired amount of air or oxygen may be admitted as described by Van Slyke and Sendroy (1932) for admission of samples, either regulated by pressure or regulated by volume. We have generally used the pressure method for regulating the approximate amount of air or oxygen introduced.

Whether air or oxygen shall be used, and how much of either, depends upon the hydrogen content of the sample. The pressure of oxygen present in the mixture burned must exceed half the pressure of the hydrogen. One can always measure an amount of

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oxygen into the chamber which will give more than 0.5  $P_S$  mm. of pressure, and be sure that a sufficient excess is present, even if the sample is pure hydrogen.

It is preferable, if the hydrogen content of the sample exceeds 10 per cent, to add pure oxygen rather than air for the combustion. One can then always take a full sized sample, with  $P_S$  of 500 mm. If air is used when the hydrogen exceeds 10 per cent, the size of the sample must be smaller. Otherwise the N<sub>2</sub> of the added air would increase the residual unburned gas, left after explosion, so much that the residual gas could not be measured in one portion.

The minimum amounts of air or oxygen that must be added, for samples of different H<sub>2</sub> and O<sub>2</sub> contents, are indicated by the following formulæ. The formulæ make allowance for any oxygen already in the sample. If the minimum required  $P_{O_2}$  or  $P_{air}$  calculated by these formulæ is zero or negative, it is obvious that enough oxygen is already present in the sample to burn the hydrogen, so that addition of oxygen is unnecessary.

$$(1) \text{ Minimum required } P_{O_2} = \left( \frac{\text{per cent H}_2}{200} - \frac{\text{per cent O}_2}{100} \right) \times P_S$$

$$(2) \text{ Minimum required } P_{air} = \left( \frac{\text{per cent H}_2}{40} - \frac{\text{per cent O}_2}{20} \right) \times P_S$$

$P_{O_2}$  = pressure of O<sub>2</sub> measured in chamber at 50 cc. volume;  $P_{air}$  = pressure of air similarly measured; per cent H<sub>2</sub> = per cent H<sub>2</sub> in gas sample; per cent O<sub>2</sub> = per cent O<sub>2</sub> in gas sample.

After the desired amount of air or oxygen has been admitted to the manometric chamber the mercury is brought to the 50 cc. mark, and reading  $p_2$  is taken on the manometer.

$$P_A = p_2 - p_0$$

$P_A$  represents the pressure at 50 cc. of the added air or oxygen.

After  $p_2$  has been noted the air or oxygen is transferred to the combustion chamber in the same manner described for transfer of the sample.

### Combustion

The manner of combustion depends upon the proportion of hydrogen in the gas mixture burned. If there is less than 10

per cent of hydrogen the mixture will burn quietly at atmospheric pressure without an explosion. If the hydrogen is between 10 and 20 per cent the mixture will burn at atmospheric pressure by explosion, the vigor of which increases with the proportion of hydrogen present. If the hydrogen is less than 20 per cent, however, the explosion is not severe enough to break the glass combustion chamber. The concentration of excess oxygen in the mixture makes no appreciable difference with the vigor of the explosion. A mixture with 9 per cent of hydrogen will burn without explosion, whether the rest of the gas is pure oxygen, or 10 per cent oxygen and 90 per cent nitrogen. And a mixture with 20 per cent of hydrogen explodes with no more apparent violence if the rest of the gas is pure oxygen than if it is nitrogen with only enough oxygen for the combustion.

Even the most explosive mixture, 2 parts of  $H_2$  to 1 of pure  $O_2$ , will burn without explosion if the pressure is so reduced that the partial pressure of hydrogen is below 70 mm. Such reduction of the hydrogen pressure is obtained by reducing the total pressure to 100 mm.

In order to decide the extent to which the ignited mixture must be attenuated to prevent explosion, the hydrogen content of the mixture is calculated from the presumed hydrogen content of the unmixed sample.

$$\text{Per cent } H_2 \text{ in mixture burned} = (\text{per cent } H_2 \text{ in sample}) \times \frac{P_S}{P_S + P_A}$$

*Combustion in One Portion at Atmospheric Pressure*—If the per cent of hydrogen in the gas mixture burned is under 15, one may safely burn the mixture in one portion. After the sample and the added oxygen have been transferred to the combustion chamber, its leveling bulb is put at such a height that the mercury in the chamber is a few mm. above the floor of the chamber, and the wire is heated. If a flash occurs the combustion is complete at once. Otherwise the wire is kept at a red glow for 10 seconds. The current is then shut off, and 30 seconds are allowed for the glass ends, into which the platinum wires are sealed, to cool so that contact with the mercury will not crack them. Then the leveling bulb of the combustion chamber is placed in the position shown in Fig. 1, the leveling bulb of the manometric chamber

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somewhat lower, and the cock at the top of the manometric chamber is turned to connect the two chambers. By opening the cock leading to the manometric leveling bulb, the greater part of the gas is drawn over into the manometric chamber. The gas is then returned to the combustion chamber in the manner previously described, in order to wash out any slight pockets or bubbles that may have failed to be transferred to the combustion chamber the first time. With the leveling bulb of the combustion chamber as shown in Fig. 1 the wire is heated again for 10 seconds to burn any traces of hydrogen that may have been washed back from the other chamber.

*Combustion in Instalments under Diminished Pressure*—If the gas mixture contains more than 15 per cent of hydrogen it is burned in two or more instalments under diminished pressure in order to prevent undesirably vigorous explosions. In this case the mixture of gas sample plus air or oxygen, after being mixed in the combustion chamber, is passed back into the manometric chamber, and the cock between the two chambers is closed. If a large sample and much oxygen or air have been taken the total gas present may exceed 50 cc. at atmospheric pressure. In such a case 50 cc. are run back into the manometric chamber, and the rest is left in the combustion chamber. The sample should not be taken so large, however, that this excess is over 10 cc. The leveling bulb of the combustion chamber is lowered to a point 660 mm. below the floor of that chamber. The leveling bulb of the manometric chamber is left level with the bottom of that chamber, and the cock connecting it with the chamber is left open. Then the cock at the top of the manometric chamber is opened to the combustion chamber until enough gas has entered the latter to cause the mercury in it to fall to within a few mm. of the floor of the chamber.

The cock connecting the two chambers is then closed, and the platinum wire is heated for 10 seconds. As the gas burns and contracts the mercury in the combustion chamber rises somewhat. After the current is turned off, at least 15 seconds are allowed for the platinum wire to cool. Then about 10 cc. more of gas are admitted from the manometric chamber, the leveling bulb of the combustion chamber being raised a little, if necessary, to keep the mercury in the chamber above the floor of the latter. The com-

bustion is then repeated as before. This process is repeated until all the gas has been transferred to the combustion chamber and submitted to combustion. The 15 seconds wait after each combustion before the cock connecting the two chambers is reopened is *never to be neglected*, because if the cock is opened while the wire is still warm, even though all glow has ceased, the stream of entering gas striking the wire may ignite, and the flash may strike back into the manometric chamber and produce an undesired explosion.

After the last combustion the gas is once run over into the manometric chamber and back to the combustion chamber, and is exposed to the heated wire again. At this ignition the leveling bulb of the combustion chamber is raised to the level of the chamber, so that the gas is under atmospheric pressure. Complete combustion of final traces of hydrogen appears to be more certain at atmospheric than at diminished pressure.

If the gas mixture contains less than 30 per cent of hydrogen it may be burned in only two portions, the first under  $\frac{1}{2}$  atmosphere pressure, the second under pressure only a little less than atmospheric.

#### *Measurement of Residual Unburned Gas*

After the last heating of the wire, and the 30 seconds interval to permit the glass ends to cool, the unburned gas is returned to the manometric chamber, followed by a little mercury. The mercury meniscus is lowered to the 50 cc. mark, and allowed to rest there 2 minutes while the gas cools. Then  $p_s$  is read. The pressure  $P_R$  of the residual unburned gas is calculated as

$$P_R = p_s - p_0$$

In case the temperature in the water jacket has changed by more than  $0.1^\circ$  since the initial  $p_0$  reading, the  $p_0$  reading is repeated after the residual gas is ejected from the chamber. The  $p_0$  changes when temperature alters the vapor tension in the chamber, the change being approximately 1.3 mm. with  $1^\circ$  of temperature. The final  $p_0$  reading is the one used to calculate  $P_R$ .

#### *Calculation*

*Temperature Correction to  $P_R$* —If the precautions outlined under "Temperature control" in Paper I of this series (Van Slyke

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and Sendroy, 1932) are observed, no significant temperature changes will occur in the chamber between the initial  $p_0$  and the  $p_2$  readings, when the operations are carried through with ordinary rapidity. At the  $p_3$  and final  $p_0$  readings after the combustion, however, the temperature may differ by 0.2° or 0.3° from the initial readings. In such cases a slight correction to the observed  $P_R$  is necessary, in accordance with the gas laws. The correction is made by the formula.

$$P_R \text{ (corrected)} = P_R \text{ (observed)} \times \frac{T_S}{T_R}$$

$T_S$  is the absolute temperature ( $t$  centigrade + 273°) at which  $P_S$  is observed, and  $T_R$  is the absolute temperature at which  $P_R$  is observed. (See p. 513, Van Slyke and Sendroy, 1932.)

*Calculation of Hydrogen*—With the  $P_R$  thus corrected, if necessary, the hydrogen pressure at 50 cc. volume is calculated as:

$$P_{H_2} = \frac{1}{3} (P_S + P_A - P_R)$$

whence

$$\text{Per cent H}_2 = \frac{100 P_{H_2}}{P_S}$$

### *Analyses by Macro Hydrogen Method*

Table I gives some experimental data and illustrates the method of calculations. The added gas for combustion was pure oxygen in all cases.

To prepare the known gas mixtures several successive portions of nitrogen and hydrogen were measured by pressure in the manometric chamber, in the same manner in which gas samples are measured. The nitrogen and hydrogen so measured were passed into a gas container over mercury, in which they were mixed, and from which samples were withdrawn for analysis.

### HYDROGEN DETERMINATION IN SMALL SAMPLES, WITH PRESSURE READINGS AT 2 CC. VOLUME

The procedure is similar to, and the calculations identical with, those described above for analyses with pressure measurements at 50 cc. volume. The same combustion chamber attached in the same manner is used.

It is especially important in analysis of the small portions of gas used, to make certain that there are no air pockets in the rubber joint between the manometric and combustion chambers. To remove any gas from this connection before an analysis is begun, the leveling bulbs of both chambers are lowered far enough to give

TABLE I  
*Macro Determination of Hydrogen on Samples of 30 to 35 Cc.*  
 Pressure measurements at 50 cc. volume

Analysis No.	Observations						Temperature	
	$p_0$ (Initial) chamber empty	$p_1$ Sample in chamber	$p_2$ $O_2$ in chamber	$p_3$ Unburned gas in chamber	$p_0$ (Final) chamber empty	Initial	Final	
	mm.	mm.	mm.	mm.	mm.	°C.	°C.	
1	21.8	455.1	610.9*	497.1	22.0	22.8	23.0	
2	21.6	483.1	567.6*	445.9	21.5	22.7	22.6	
3	22.6	591.1	221.0	577.9	22.3	23.6	23.4	
4	22.2	563.2	203.9	544.9	22.1	23.3	23.4	
5	22.0	575.9	133.0	481.7	21.9	23.2	23.3	
6	19.9	565.4	368.7	97.2	20.6	21.0	21.5	

Analysis No.	Calculations								$H_2$ present per cent
	$P_S = p_1 - p_0$ (initial)	$P_A = p_2 - p_0$ (initial)	$P_S + P_A$	$P_R$ observed = $p_3 - p_0$ (final)	$P_R$ corrected to initial temperature	$Contraction = \frac{P_S + P_A}{P_R}$	$P_{H_2} = \frac{1}{2}$ con- traction	$H_2$ found = $\frac{100 P_{H_2}}{P_S}$	
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	per cent	
1	433.3	589.1*	475.1	474.8	114.3	76.2	17.58	17.56	
2	461.5	546.0*	424.4	424.5	121.5	81.0	17.56	17.56	
3	568.5	198.4	766.9	555.6	556.0	210.9	140.6	24.72	24.73
4	541.0	181.7	722.7	522.8	522.6	200.1	133.4	24.68	24.73
5	553.9	111.0	664.9	459.8	459.6	205.3	136.9	24.72	24.73
6	545.5	348.8	894.3	76.6	76.5	817.8	545.2	99.95	100.00

\* In Analyses 1 and 2 the oxygen was measured into the manometric chamber without first removing the gas sample. Hence in these two analyses the value  $P_S + P_A$  is observed directly as  $p_2 - p_0$ .

a Torricellian vacuum in the upper part of each. The bulb of the combustion chamber is then raised so that mercury fills that chamber and passes over into the manometric chamber. Any gas bubble previously caught in the connections will be greatly

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expanded by this procedure and swept into the manometric chamber, from which it is ejected.

The  $p_0$  reading is determined as in the macro method, except that the meniscus of the mercury is at the 2 cc. mark instead of the 50 cc. one.

The sample is admitted most conveniently from a modified Hempel pipette as described by Van Slyke and Hiller (1928). The sample is admitted with the leveling bulb of the manometric chamber at the medium position, level with the bottom of the chamber, and with the cock open between this bulb and the chamber, so that the gas in the chamber is under slightly less than atmospheric pressure. About 1.5 cc. of sample observed at this pressure is admitted in one portion into the chamber.  $p_1$  is then read with the mercury at the 2 cc. mark in the chamber. It is essential for accuracy that a reading glass be used to observe both the mercury meniscus in the chamber and that in the manometer. As in the macro analysis,  $P_s = p_1 - p_0$ .

The principles governing the measurement of the amount of oxygen or air to add are the same as in the macro analysis. The oxygen or air pressure is measured as  $p_2 - p_0$ , as in the macro analysis, but at 2 cc. volume.

The combustion of the mixed gases is always done at one time, even with the most explosive mixtures. The amount of gas is so small that it does not more than half fill the chamber even when the pressure has been reduced to 100 mm. Even when the mixture does not contain enough hydrogen to be explosive the combustion must be carried out under reduced pressure, merely to expand the gas to sufficient volume to fill the upper part of the combustion chamber, where the wire is.

After the ignition the chamber is allowed to cool for 30 seconds before the mercury is permitted to rise and touch the hot glass tips into which the platinum wire is sealed. The residual gas is then passed into the manometric chamber and back into the combustion chamber, where it is ignited again for 10 seconds. This process is repeated once more, and the gas is ignited a third and final time. The residual gas is then returned to the manometric chamber and its pressure is taken at 2 cc. volume.

The object of the extra passage and ignition is to insure that the last traces of gas which may be trapped in the joint between

the two chambers shall be mixed with the other gases and ignited.

In order to prevent the trapping of any of the residual gas in the joint during the transfer back to the manometric chamber after the final combustion, the leveling bulb of the combustion chamber is left at the low level, 660 mm. below the floor of that chamber,

TABLE II  
*Micro Determination of Hydrogen on Samples of about 1 Cc.  
Pressure measurements at 2 cc. volume*

Analysis No	Observations						Temperature
	$p_0$ (Initial) chamber empty	$p_1$ Sample in chamber	$p_2$ $O_2$ in chamber	$p_3$ Unburned gas in chamber	Initial	Final	
	mm	mm	mm	mm	°C	°C.	
1	107.0	498.5	586.3*	482.1	22.4	22.5	
2	107.9	538.1	594.9*	483.0	23.2	23.2	
3	107.7	572.1	175.3	467.6	23.6	23.6	
4	107.7	568.0	202.5	490.8	23.5	23.5	
5	107.0	576.3	211.9	506.7	23.1	23.1	

Analysis No	Calculations								$H_2$ present
	$P_S = p_1 - p_0$	$P_A = p_2 - p_0$	$P_S + P_A$	$P_R$ from ob- served $p_2 - p_0$	$P_R$ corrected to initial temperature	$\frac{P_S + P_A}{P_R} - 1$	$P_{H_2} = \frac{1}{2}$ con- traction		
	mm	mm	mm	mm	mm	mm	per cent		
1	391.5	479.3*	375.1	375.0	104.3	69.5	17.74	17.56	
2	430.2	487.0*	375.1	375.1	111.9	74.6	17.33	17.56	
3	464.4	67.6	532.0	359.9	359.9	172.1	114.7	24.70	24.73
4	468.3	94.8	555.1	383.1	383.1	172.0	114.7	24.90	24.73
5	469.3	104.9	574.2	399.7	399.7	174.5	116.4	24.78	24.73

\* In Analyses 1 and 2 the oxygen was measured into the manometric chamber without first removing the gas sample. Hence in these two analyses the value  $P_S + P_A$  is observed directly as  $p_2 - p_0$ . In this series  $p_0$  final was assumed to be the same as  $p_0$  initial, since the temperature change during the analysis in no case exceeded 0.1°.

and the manometric chamber is partially evacuated before the cock between the two chambers is reopened. The cock between the manometric chamber and its leveling bulb is then closed, the

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two chambers are connected, and the highly rarified gas in the combustion chamber, followed by some mercury, is run into the manometric chamber.

The temperature corrections and method of calculation are identical with those described for the macro analysis. Here, however, the temperature corrections are not so significant because the relative error of the other measurements is greater.

In Table II are given the results of some analyses, and illustrations of the calculations.

### *Determination of Oxygen*

Either the macro or the micro method above described for the determination of hydrogen serves equally well for determination of oxygen if the combustion is carried out with excess of hydrogen.

Because the contraction on ignition is three times the oxygen volume, the error in oxygen analysis is half that in hydrogen determinations. For oxygen, results are exact within 0.02 or 0.03 volume per cent, about the same as in analyses with the 10 cc. Haldane apparatus.

In three points the procedure for oxygen determination differs from that for hydrogen. (1) For each volume of oxygen present 2 of hydrogen must be added. Hence if the oxygen content of the sample is over 50 per cent the  $P_s$  of the sample must be less than 500 mm. if one is to avoid measuring the hydrogen in two installations. (2) Instead of 10 seconds heating of the wire for each combustion, 30 seconds are needed when the residual gas contains a large proportion of hydrogen. (3) The minimum explosive concentration of oxygen is somewhat less than that of hydrogen. Hydrogen, with an excess of O<sub>2</sub>, is explosive in more than 9 per cent concentration at atmospheric pressure, or when the H<sub>2</sub> partial pressure exceeds 70 mm. Oxygen, with an excess of H<sub>2</sub>, is explosive in more than 7 per cent concentration at atmospheric pressure, or when the O<sub>2</sub> partial pressure exceeds 50 mm.

The procedures for measuring the gas sample, adding the gas required for combustion (hydrogen in this case), carrying out the combustion, and measuring the residual gas, are identical with those described above for determination of hydrogen, except that 30 seconds instead of 10 seconds are given to the ignition.

The amount of hydrogen, measured by its pressure taken at the same volume as  $P_S$ , is estimated as:

$$\text{Minimum required } P_{H_2} = \frac{\text{per cent O}_2 \text{ in sample}}{50} \times P_S$$

For atmospheric air, with 21 per cent oxygen,  $P_{H_2}$  must be at least  $\frac{21}{50} \times P_S = 0.42 P_S$ . The air-hydrogen mixture, to avoid explosions, should be burned in three portions, in macro analyses.

### *Calculations*

The calculations resemble those in the hydrogen determinations, except that here  $P_{H_2}$  replaces  $P_A$  in the hydrogen analyses, and the value of  $P_{O_2}$  is calculated as one-third instead of two-thirds of the contraction.

$$P_{O_2} = \frac{1}{3} (P_S + P_{H_2} - P_R)$$

$$\text{Per cent O}_2 = \frac{100 P_{O_2}}{P_S}$$

### *Determinations of Oxygen in Outdoor Air*

Illustrative results are given in Table III. In these air analyses the amount of hydrogen admitted was such that its pressure was somewhat over 50 per cent of the sample pressure, to insure an excess of hydrogen, of which the required amount is 42 per cent of the air sample.

### *Determination of Both Hydrogen and Oxygen in Same Gas Mixture*

For this purpose the sample is first ignited without addition of either gas. Then the residual gas is mixed with either hydrogen or oxygen, to burn the remainder of whichever of the two gases was in excess in the sample, and a second combustion is performed on the mixture.

### *Combustible Organic Gases*

Methane, acetylene, and other organic gases determinable by combustion can undoubtedly be determined in the same manner as hydrogen, the  $CO_2$  formed being determined when necessary, by

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applying gas methods previously described (Van Slyke and Sendroy, 1932; Van Slyke, Sendroy, and Liu, 1932) to the ignited gas mixture.

TABLE III  
*Macro Determinations of Oxygen in Outdoor Air*  
Pressure measurements at 50 cc. volume

Analysis No.	Observations						Temperature	
	$p_0$ (Initial) chamber empty	$p_1$ Sample in chamber	$p_2$ H <sub>2</sub> in chamber	$p_3$ Unburned gas in chamber	$p_0$ (Final) chamber empty	Initial	Final	
	mm.	mm.	mm.	mm.	mm.	°C.	°C.	
1	23.4	550.1	292.8	490.3	23.9	24.7	25.0	
2	24.2	567.9	351.3	554.3	24.2	25.3	25.3	

Analysis No.	Calculations								O <sub>2</sub> present (as- sumed for outdoor air)
	$P_S = p_1 - p_0$	$P_{H_2} = p_2 - p_0$	$P_S + P_{H_2}$	$P_R$ from ob- served $p_1$ - $p_0$ final	$P_R$ corrected to initial temperature	$\frac{P_S + P_{H_2}}{P_R}$	$P_{O_2} = \frac{1}{4}$ con- traction	$O_2$ found = $\frac{100 P_{O_2}}{P_S}$	
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	per cent	per cent
1	526.7	269.4	796.1	466.4	465.9	330.2	190.1	20.90	20.93
2	543.7	327.1	870.8	530.1	530.1	340.7	113.6	20.90	20.93

\* The oxygen content of New York air is sometimes a little below 20.93, because of the amount of coal burned.

## SUMMARY

Methods are described for determination of hydrogen and oxygen in gas mixtures by the combustion procedure, the gases before and after ignition being measured by pressure in the manometric apparatus of Van Slyke and Neill. The apparatus can be used for micro determinations, with 1.5 cc. samples of gas, or for macro analyses, with samples of 30 to 35 cc. By reducing the pressure in the combustion chamber even the most explosive mixtures can be burned smoothly. They do not need to be diluted with inert gas as in the usual methods. The maximum error in micro determinations is about  $\pm 0.2$  per cent of the total gas. In macro determinations of hydrogen the maximum error is  $\pm 0.05$  per cent, and in oxygen determinations  $\pm 0.03$  per cent of the total gas.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### V. HYDROGEN BY ABSORPTION WITH PAAL'S PICRATE-PALLADIUM SOLUTION

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The method here described is especially adapted to micro analyses of gas samples of less than 2 cc. volume. It requires about the same time, 20 minutes, as hydrogen determination by the combustion method described in the preceding paper (Van Slyke and Hanke, 1932) and has the same accuracy, about 0.2 volume per cent, as the combustion method with similar small samples of gas. The palladium method has the advantage that it requires no combustion chamber or other accessory apparatus. It has the limitations that it cannot be economically used for more accurate analyses of larger samples of gas, and that preliminary removal of oxygen and carbon dioxide is necessary.

The use of a solution of sodium picrate and colloidal palladium for absorption of hydrogen in gas analysis was introduced by Paal and Hartmann (1910), and has since been used by others (Brunck, 1910; Van Slyke and Binger, 1923).

The general technique for temperature control, admission of samples, etc., discussed in the introductory paper of this series (Van Slyke and Sendroy, 1932) is to be observed in carrying out the operations described below.

#### *General Procedure*

A sample of 1 to 1.5 cc. of the gas mixture, previously freed from oxygen and carbon dioxide, is let into the reaction chamber and measured by the pressure exerted at 2 cc. volume. The hydrogen is then absorbed with 0.2 cc. of a palladium-sodium picrate solution.

Because of gradual inactivation of the palladium by the metallic mercury, exposure of the gas to the solution in the chamber is accomplished by a special manipulation to minimize the contact of the palladium with the mercury. After the absorption of the hydrogen is complete, the pressure of the residual gas is read at 2 cc. volume, and the hydrogen is calculated from the decrease in pressure.

The manometric apparatus used is that previously described. (Van Slyke and Neill, 1924; Van Slyke, 1927).

#### *Reagents*

*Palladium-Sodium Picrate Solution*<sup>1</sup>—This solution contains 2 per cent of colloidal palladium “nach Paal” (Paal and Hartmann, 1910) and 3.5 per cent picric acid in 0.154 N NaOH. To 3.5 gm. of picric acid, 15.4 cc. of N NaOH and about 50 cc. of water are added, and the mixture is warmed to about 50° until the picric acid is dissolved. Then 2 gm. of colloidal palladium, previously stirred up with about 20 cc. of water, are added, and the whole is made up to 100 cc. It takes about 1 hour with occasional stirring for the palladium solution to become entirely homogeneous.

Since colloidal palladium is gradually inactivated by contact with metallic mercury, it is necessary to avoid introducing mercury into the reagent accidentally. The stock reagent should be kept in a stoppered bottle at a distance from the manometric apparatus in order to avoid contamination with the mercury droplets that are likely to pervade the surroundings. A portion of reagent sufficient for the day's analyses is withdrawn into a small flask.

Paal and Hartmann (1910) used a palladium solution containing 5 per cent picric acid, instead of the 3.5 per cent here described. We have found that from the 5 per cent solution a considerable amount of sodium picrate gradually precipitates on standing, and that by decreasing the concentration to 3.5 per cent, this difficulty can be avoided.

If the picric acid is reduced to triaminophenol by the hydrogen, 0.2 cc. of the reagent combines with 5.5 cc. of hydrogen measured

<sup>1</sup> The colloidal palladium was purchased from Dr. Theodor Schuchardt, Chemische Fabrik, Görlitz, Germany, for \$8.25 a gm. When 0.2 cc. of a 2 per cent solution, or 4 mg., is used, the palladium costs about 3 cents for each analysis.

at 1 atmosphere pressure. The method as described therefore provides theoretically a 4-fold excess of reagent, even when pure hydrogen is analyzed, and in practice has been found adequate for analysis of pure hydrogen.

*Gas-Free Water*—Distilled water is deaerated by the method described by Van Slyke and Neill (1924, p. 534) for the preparation of gas-free reagents. It is then stored in a Hempel pipette over mercury as described by Van Slyke and Hiller (1928, p. 809).

#### *Measurement of Gas Sample*

The gas sample is conveniently stored in a modified Hempel pipette described by Van Slyke and Hiller (1928, p. 809). As displacement liquid in the pipette one may use alkaline hyposulphite or pyrogallol solution, which absorbs the CO<sub>2</sub> and O<sub>2</sub> from the gas mixture and thereby prepares it for H<sub>2</sub> determination by this method. The solution in the upper bulb of the Hempel pipette is covered with a layer of oil.

The sample of about 1.5 cc. of gas is admitted into the reaction chamber in the manner described for "Admission of sample regulated by volume" on p. 516 of Paper I of this series (Van Slyke and Sendroy, 1932).

The measurement of the gas sample is carried out as described in Paper I (Van Slyke and Sendroy, 1932),  $p_0$  and  $p_1$  being observed, before and after admission of the gas sample, with the mercury meniscus in the chamber at the 2 cc. mark. The pressure  $P_S$  exerted by the gas sample at 2 cc. volume is calculated as:

$$P_S = c_1 (p_1 - p_0)$$

The correction factor,  $c_1$ , which has a value of about 1.01, is discussed below.

#### *Absorption of Hydrogen with Picrate-Palladium Solution*

The leveling bulb of the manometric chamber is placed in its ring level with the bottom of the chamber, and the cock between leveling bulb and chamber is left open, so that the gas in the chamber is under slight negative pressure.

Excess of mercury is removed from the cup of the chamber, so that there is just a little more than necessary to fill the capillary.

0.2 cc. of the palladium-sodium picrate reagent, measured with a pipette to within 0.01 cc., is introduced into the cup. With a thin wire previously dipped into caprylic alcohol, any bubble of air between the palladium solution and the mercury is removed. Now one-fifth, roughly estimated, of the 0.2 cc. of solution is admitted into the reaction chamber. An interval clock is set for a 10 minute period. If the drop of palladium solution admitted remains trapped by capillarity in the top of the reaction chamber, it is necessary to tap the chamber by hand in order to cause the reagent to flow down its walls. After about 10 seconds, when most of the liquid has flown down over the mercury, the stop-cock between the leveling bulb of the apparatus and the gas chamber is closed, and the leveling bulb is lowered to about 76 cm. below the chamber. The cock leading to the leveling bulb is then opened quickly, to let the mercury in the chamber fall 2 or 3 cm. below the 2 cc. mark. The cock is then closed and the bulb is lifted to its ring in the medium position, level with the bottom of the chamber. When the mercury falls the palladium solution is left distributed more or less uniformly over the glass walls of the chamber, and as it gradually drains down after the mercury a large surface of the palladium solution is exposed to the gas phase. After 20 or 30 seconds, when most of the palladium solution has drained down to the mercury surface, the leveling bulb cock is opened slightly, allowing the mercury to rise slowly as far as it will go, pushing almost all of the palladium solution ahead of it. The quick lowering and slow raising of the mercury in the chamber is then once repeated.<sup>2</sup>

The 0.16 cc. of reagent left in the cup is admitted in four instalments. After each addition of reagent the mercury in the chamber is lowered and raised twice in the manner described above. About 2 minutes should be taken for the admission and manipulation of each instalment, so that 10 minutes are taken for complete absorp-

<sup>2</sup> If the mercury were allowed to rise rapidly most of the palladium solution would remain adherent to the glass walls of the chamber. Consequently a large surface of the solution would be exposed to the mercury, and the palladium would be rapidly inactivated. If the mercury rises gradually, in the course of about 10 seconds, practically all of the palladium solution drains upward ahead of the mercury, thus making minimum contact with the mercury surface.

tion of the hydrogen. After the last instalment of solution has been run into the chamber, except the small amount necessary to fill the capillaries of the stop-cock and cup, the cup is half filled with water, and the stop-cock is sealed with mercury.

Admission of 0.04 cc. portions of the palladium solution can, with practice, be managed with sufficient accuracy by means of the well greased cock of the manometric chamber. During the admission the leveling bulb of the chamber is about level with the bottom of the latter, and the cock between chamber and bulb is open, so that the gas in the chamber is under slight negative pressure.

Another procedure, which requires less skill, is the following. The leveling bulb is so held that the mercury surface in it is 2 cm. lower than in the chamber, and the cock between chamber and bulb is then closed, so that the gas in the chamber is under 20 mm. of negative pressure. The cock at the top of the chamber is now opened, and an amount of palladium solution automatically limited to about 0.04 cc. flows into the chamber. This procedure is applicable only with the closed manometer type of apparatus.

#### *Addition of Air-Free Water and Measurement of Residual Gas*

After the hydrogen has been absorbed, 2 to 2.5 cc. of gas-free water are admitted to the chamber. The water dilutes the palladium solution so that it drains more readily, and so that it becomes transparent and its meniscus thus more accurately definable.\*

The water is admitted into the chamber from a modified Hempel pipette in the manner described by Van Slyke and Hiller (1928, pp. 810 and 811), for admission of CO, except that here the pipette contains water stored over mercury instead of gas over water. It is not necessary to measure the water added more accurately than to keep its volume between 2.0 and 2.5 cc. This volume can usually be estimated with enough accuracy from the space the water occupies in the upper part of the chamber. If the amount

\* It is not sufficient here to use gas-free water which is stored under oil, nor is it permissible to run the water into the open cup, and from there into the chamber. Even momentary exposure of the water to the atmosphere permits absorption of sufficiently large and variable amounts of air to affect the accuracy of the analysis. It is necessary to store the air-free water over mercury, and to deliver it from its container directly through a mercury seal into the reaction chamber without contact with air.

of water is not thus approximately controlled, a measurable error may be introduced because of different quantities of the residual gas later dissolved by the aqueous phase.

After the water is admitted the Hempel pipette is removed and the stop-cock is sealed with mercury. The mercury meniscus is then lowered to the 50 cc. mark and the chamber is shaken for 2 minutes. Thereby slight amounts of nitrogen gas which have been absorbed by the water during its admission are almost completely returned to the gas phase. (Only about 0.001 of the total N<sub>2</sub> in the chamber remains dissolved under the conditions of equilibration.) The water meniscus is then brought to the 2 cc. mark and the manometer reading  $p_2$  is taken. The temperature is recorded.

The gases are ejected without loss of liquid as described in a previous paper (Van Slyke, 1926-27, p. 240). The stop-cock is sealed with mercury, the solution meniscus is brought exactly to the 2 cc. mark, and the manometer reading,  $p_3$ , is taken. Expressing the pressure of the residual gas as  $P_R$ , we have

$$P_R = p_2 - p_3 - c_2$$

The  $p_3$  reading ends the analysis.

#### *Determination of $c_1$ Correction*

The fact that the difference in curvature of menisci makes the volume of gas over a mercury meniscus at the 2 cc. mark in the chamber exceed by about 1 per cent the volume over a water meniscus at the same mark, has been discussed by Van Slyke and Sendroy (1932). Since  $P_S$  is measured with the gas over mercury and  $P_R$  with it over water, a correction must be made to  $P_R$  for the above mentioned volume difference in order to make  $P_R$  accurately comparable with  $P_S$ . There is also a slight additional correction to  $P_R$  required for the slight amount of N<sub>2</sub> that is dissolved by the 2.2 to 2.7 cc. of water in the chamber. After this water has been shaken *in vacuo* as directed for the last stage of the analysis, about 0.001 of the N<sub>2</sub> in the chamber remains in solution. And when the gas volume is diminished again to 2 cc. a little more N<sub>2</sub> dissolves, so that the total dissolved is about 0.002 of that present.

The correction,  $c_1$ , for the combined effects of the difference between mercury and water menisci, and for dissolved N<sub>2</sub>, is

determined as follows: A reading,  $p_0$ , is taken with no gas or visible water in the chamber, and with the mercury meniscus at the 2 cc. mark. Then enough air is admitted to exert 400 to 500 mm. pressure at 2 cc. volume, and  $p_1$  reading is taken, again over mercury. 2 cc. of air-free water are now admitted to the chamber, the mercury is lowered to the 50 cc. mark, and the chamber is shaken for 2 minutes. The water meniscus is now brought to the 2 cc. mark and the reading  $p_2$  is taken. Finally the air is ejected from the chamber, without loss of more than 0.1 cc. of the water, and the reading  $p_3$  is taken with the chamber gas-free and the water meniscus again at the 2 cc. mark. Then

$$c_1 = \frac{p_2 - p_3}{p_1 - p_0} = \frac{\text{pressure over water}}{\text{pressure over mercury}} = \text{about } 1.01$$

The exact value of  $c_1$  is determined by repeating several times the above determination and taking the average result. The value

TABLE I  
*Determination of  $c_1$  for Authors' Apparatus*  
Pressure readings at 2 cc. volume

Analysis No.	$p_0$	$p_1$	$p_2$	$p_3$	$c_1 = \frac{p_2 - p_3}{p_1 - p_0}$	Temper-ature at $p_1$ reading	Temper-ature at $p_2$ reading
	mm.	mm.	mm.	mm.		°C.	°C.
1	105.0	517.3	515.2	99.0	1.0095	20.9	21.0
2	109.2	545.0	525.5	86.1	1.0082	24.9	25.0
3	106.8	536.4	519.4	85.6	1.0097	22.4	22.5

of  $c_1$  can be thus determined with a precision of about 1 part per 1000 (see Table I).

*Correction,  $c_2$ , for Air Carried into Chamber Dissolved in 0.2 Cc. of Palladium Solution*—The palladium solution when admitted is saturated with air, which it gives off in the chamber. To determine  $c_2$ , 1 cc. of the palladium solution and 1 cc. of completely air-free water are measured into the chamber. The mercury is lowered to the 50 cc. mark and the solution is extracted by shaking for 2 minutes. The pressure,  $p_1$ , is then measured at 2 cc. volume. The gas is ejected, and  $p_2$  is read.

$$c_2 = 0.2 (p_1 - p_2)$$

V. Manometric H<sub>2</sub> by Absorption

In this case 5 times the usual amount of palladium solution is taken and the pressure of the extracted air is divided by 5 to obtain the correction for the amount of reagent used in the analyses. The  $c_2$  correction is usually about 1.5 mm.

*Correction for Temperature Changes*—If the temperature in the water jacket of the chamber changes during the interval of 10 or 12

TABLE II  
*Analyses of Hydrogen-Nitrogen Mixture Containing 17.56 Per Cent Hydrogen*  
Pressure readings at 2 cc. volume

Analysis No.	Observations					
	$p_0$ Chamber empty	$p_1$ Sample in chamber	$p_2$ After H <sub>2</sub> absorption	$p_1$ Reagent solution in chamber	Temperature at $p_1$ reading	Temperature at $p_2$ reading
1	mm.	mm.	mm.	mm.	°C.	°C.
107.0	543.8	449.2	84.3	22.7	22.7	
2	112.0	548.3	451.3	86.9	23.5	23.6
3	108.0	573.0	474.1	85.9	23.6	23.9
4	107.7	500.3	413.2	85.0	23.1	23.3

Analysis No.	Calculations					
	$P_S$		$P_R$		$P_{H_2} = P_S - P_R$	H <sub>2</sub>
Uncorrected, $p_1 - p_0$	Corrected by $c_1$ of 1.009	Uncorrected, $p_2 - p_0$	Corrected by $c_2$ of 1.5 mm.	mm.	per cent	
mm.	mm.	mm.	mm.	mm.		
1	436.8	440.7	364.9	363.4	77.3	17.54
2	436.3	440.2	364.4	362.9	77.3	17.54
3*	465.0	469.2	388.2	386.3	82.9	17.65
4	392.6	396.1	328.2	326.7	69.4	17.52

\* In Analysis 3,  $\frac{8}{500}$  of  $p_2 - p_0$  is deducted for temperature correction. The temperature changes in the other analyses are not significant.

minutes between the  $P_S$  and  $P_R$  readings, the  $P_R$  reading is multiplied by the factor,  $\frac{T_S}{T_R}$ , in which  $T_S$  and  $T_R$  represent the absolute temperatures at the readings of  $P_S$  and  $P_R$ , respectively. (See Equation 1 and discussion of temperature corrections in Paper I of this series (Van Slyke and Sendroy, 1932, p. 513).)

*Calculation**A. Hydrogen in CO<sub>2</sub>- and O<sub>2</sub>-Free Gas Analyzed*

$$P_{H_2} = P_S - P_R$$

$$\text{Per cent H}_2 = 100 \times \frac{P_{H_2}}{P_S}$$

*B. Hydrogen in Original Gas Containing CO<sub>2</sub> and O<sub>2</sub>*

$$\text{Per cent H}_2 = A \times \frac{100}{100 - (CO_2 + O_2)}$$

*A* = per cent of H<sub>2</sub> calculated by Formula A; (CO<sub>2</sub> + O<sub>2</sub>) = per cent of CO<sub>2</sub> + O<sub>2</sub> in original gas mixture. The value of (CO<sub>2</sub> + O<sub>2</sub>) must be determined by separate analysis if the H<sub>2</sub> content of the original gas mixture is desired.

*Analyses of Known Mixtures of Hydrogen and Nitrogen*

Known mixtures of the two gases were prepared by running successive portions of about 30 cc. of the gases into the chamber of

TABLE III

*Analyses of Hydrogen-Nitrogen Mixture Containing 49.88 Per Cent Hydrogen*  
Pressure readings at 2 cc. volume

Analysis No.	Observations					
	p <sub>0</sub> Chamber empty	p <sub>1</sub> Sample in chamber	p <sub>2</sub> After H <sub>2</sub> absorption	p <sub>3</sub> Reagent solution in chamber	Temper- ature at p <sub>1</sub> reading	Temper- ature at p <sub>2</sub> reading
1	mm. 103.3	mm. 482.1	mm. 280.0	mm. 85.1	°C. 20.6	°C. 21.3
2	mm. 105.4	mm. 482.5	mm. 280.1	mm. 86.8	°C. 22.5	°C. 22.5
Calculations						
Analysis No.	P <sub>S</sub>		P <sub>R</sub>		P <sub>H<sub>2</sub></sub> = P <sub>S</sub> - P <sub>R</sub>	H <sub>2</sub>
	Uncor- rected, p <sub>1</sub> - p <sub>0</sub>	Corrected by c <sub>1</sub> of 1.009	Uncor- rected, p <sub>2</sub> - p <sub>1</sub>	Corrected by c <sub>2</sub> of 2.6 mm.	Corrected to tem- perature of P <sub>S</sub>	per cent
1	mm. 378.8	mm. 382.2	mm. 194.9	mm. 192.3	mm. 191.9	49.78
2	mm. 377.1	mm. 380.5	mm. 193.3	mm. 190.7	mm. 190.7	49.90

V. Manometric H<sub>2</sub> by Absorption

the manometric apparatus, measuring the pressures at 50 cc. volume, and transferring the portions to gas containers over mercury. In Tables II to IV are given the analyses of two mixtures so prepared and of a sample of commercial hydrogen.

*Absorption of Hydrogen in Presence of Oxygen*

The presence of oxygen in amounts equivalent to the hydrogen (1 part of oxygen to 2 parts of hydrogen) markedly decreases the

TABLE IV  
*Analyses of Commercial Hydrogen*  
Pressure readings at 2 cc. volume

Analysis No.	Observations					
	$p_0$ mm.	$p_1$ mm.	$p_2$ mm.	$p_3$ mm.	Temperature at $p_1$ reading °C.	Temperature at $p_2$ reading °C.
1	107.5	512.0	8.91	8.75	24.5	24.5
2	104.8	509.0	8.69	8.53	22.0	22.0
3	105.2	431.8	8.71	8.60	22.6	22.6

Analysis No.	Calculations					
	$P_S$		$P_R$		$P_{H_2} =$ $P_S - P_R$	$H_2$
Uncorrected, $p_1 - p_0$	Corrected by $c_1$ of 1.009	Uncorrected, $p_2 - p_3$	Corrected by $c_2$ of 1.5 mm.	mm.	per cent	
1	404.5	408.1	1.6	0.1	408.0	99.98
2	404.2	408.5	1.6	0.1	408.7	99.98
3	326.6	329.5	1.1	-0.4	329.9	100.12

rate of hydrogen absorption by the reagent, and makes the absorption very incomplete in the 10 minute period above prescribed. Larger amounts of oxygen further decrease the rate of hydrogen absorption. On the other hand, in the presence of a great excess of hydrogen, small quantities of oxygen are partially absorbed along with all of the hydrogen by the above described procedure. This inconstant behavior of oxygen makes it necessary to remove oxygen before attempting the absorption of hydrogen by the methods described. Table V gives the results of analyses in the presence of oxygen.

$\text{CO}_2$  must also be removed before the analysis, because it would be absorbed by the alkaline palladium solution.

TABLE V

*Results of Attempts to Determine Hydrogen by Absorption with Picrate-Palladium Solution in Presence of Oxygen*

Analysis No.	Composition of gas mixtures used			Result of $\text{H}_2$ analysis	$\frac{\text{H}_2 \text{ found}}{\text{H}_2 \text{ present}}$
	Hydrogen per cent	Oxygen per cent	Nitrogen per cent		
1	17.56	0.00	82.44	17.56	100.00
2	15.70	10.56	73.74	6.62	42.15
3	100.0	0.00	0.00	99.88	99.88
4	86.05	13.95	0.00	99.58	115.6*
5	67.21	32.79	0.00	49.10	73.02
6	63.27	36.73	0.00	36.79	58.14

\* Greater than 100 because of absorption of some oxygen along with all of the hydrogen.

## SUMMARY

A method is described for the quantitative estimation of hydrogen in gas mixtures by absorption with a colloidal palladium-sodium picrate solution in the manometric gas apparatus of Van Slyke and Neill. A complete analysis can be performed in 20 minutes. With samples of 1.5 cc. the maximum error is 0.2 per cent of the total gas.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### VI. CARBON MONOXIDE BY ABSORPTION WITH BLOOD

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For determination in air of small amounts of CO, such as are encountered in physiological experiments or as atmospheric contaminations, a principle employed by Arnold, Carrier, Smith, and Whipple (1), and by others (2-5) is, with certain refinements, again applied in this paper. The O<sub>2</sub> is removed, and from the N<sub>2</sub>-CO residue the CO is absorbed by reduced blood. The absorbed CO in the blood is then measured by the technique of blood gas analysis. The latter is carried out, with slight modification, according to the method of Sendroy and Liu (6) for the determination of CO in blood. In the absence of oxygen, the affinity of reduced ox blood for carbon monoxide is so great that, even when the concentration of CO in the gas is only 0.05 per cent, it is absorbed by the blood, under the conditions of this analysis, to the extent of 93.7 + 2.4 per cent.

Carbon monoxide in air, in concentrations of from 0.05 to 0.3 volume per cent, can be determined with a maximum error of  $\pm 5$  per cent of the amount present. The CO estimation can conveniently be combined with determination of oxygen by the method of Van Slyke and Sendroy (7), in the same air sample.

#### *Reagents*

With the exception of the air-free 1 N NaOH, which is omitted here, the reagents required are those used by Sendroy and Liu ((6) p. 134). However, it has been found advantageous to increase the volume of concentrated lactic acid from 8 to 10 cc. per liter in the preparation of the acid ferricyanide reagent. In addition, the following solutions are prepared.

(a) *Sodium hyposulfite, 20 per cent solution with anhydrous*

*quinone sulfonate catalyst*, for absorption of O<sub>2</sub> from air, the preparation of which is described by Van Slyke ((8) p. 124). This Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> solution, according to Sendroy (9) may most conveniently be kept over mercury.

(b) *Sodium hyposulfite, 20 per cent solution without catalyst*, for blood reduction, is also prepared as above, 10 cc. at a time, and is kept under oil for the day's work.

(c) *Fresh ox blood* is reduced as described below.

#### Procedure

*Measurement and Deoxygenation of Gas Sample*—The cleaned chamber of the manometric apparatus of Van Slyke and Neill (10) is connected through the capillary side arm at the top with a gas sample container of about 40 or 50 cc. capacity, with leveling bulb attached, as shown in Fig. 1 of Van Slyke, Sendroy, and Liu (11). The container is filled with mercury, as are the connecting tubes, so that no gas remains in the system. For the connection, a rubber tube with heavy walls and small bore is used. The tube should be just long enough to permit shaking of the chamber without inconvenient tugging on the gas container.

From another container the sample of gas, about 35 cc., is admitted into the chamber through a mercury seal in the cup, as shown in Fig. 1 of Van Slyke and Sendroy (7). The manometric readings,  $p_0$  and  $p_1$ , are taken as described by Van Slyke and Sendroy, before and after admission of the gas sample, and the sample is measured by its pressure,  $P_S$ , at 50 cc. volume.

$$P_S = p_1 - p_0$$

The sample usually taken for this work is such that  $P_S$  is about 500 mm. at room temperature.

To remove oxygen (and CO<sub>2</sub>) from the sample, the latter is shaken with 3.0 cc. of alkaline hyposulfite solution as described on p. 526 of Van Slyke and Sendroy (7) for determination of N<sub>2</sub> in air.<sup>1</sup> The oxygen-free gas in the chamber is then run through the

<sup>1</sup> If the O<sub>2</sub> + CO<sub>2</sub> content of the gas sample is desired, a reading  $p'_1$  is taken immediately after the deoxygenation of the gas sample, with the mercury meniscus at the 50 cc. mark in the chamber, the 3 cc. of hyposulfite solution over the mercury, and the unabsorbed N<sub>2</sub> + CO in the 47 cc. gas space.

chamber's capillary outlet into the mercury-filled container already attached. The hyposulfite is permitted to follow the gas into the narrow upper part of the chamber until the solution *just* touches the stop-cock.<sup>2</sup> The stop-cock is then turned, the hyposulfite is ejected into the cup above the chamber, and the chamber is cleaned with water. The deoxygenated gas sample is kept in the attached container under *slightly* positive pressure, until ready for equilibration with the reduced blood.

*Removal of Air from Ferricyanide Reagent*—1 drop of caprylic alcohol and 15 cc. of the acid ferricyanide reagent are run into the chamber of the manometric apparatus and freed of air. The dissolved air is removed by evacuating the chamber, shaking 3 minutes, ejecting the extracted air, and repeating the process once more. The gas-free reagent is then forced up into a 25 cc. burette in the manner described by Van Slyke and Neill ((10) p. 535) for the handling and storage of air-free reagents under oil. The burette should contain sufficient oil to make a layer of at least 10 cm. deep, and the tip, which must extend long enough to reach the bottom of the chamber cup, is provided with a rubber ring to make a tight connection. The burette is set aside until the reduced blood has been prepared for the analysis. A fresh portion of reagent must be freed of air for each analysis, because the layer of oil prevents reabsorption of significant amounts of air for only about a half hour.

*Preparation of Reduced Blood*—The manometric chamber is washed, and a 5 cc. portion ( $\pm$  0.1 cc.) of ox blood, of normal hemoglobin content, is run into the cleaned chamber. 2 drops of caprylic alcohol are added, and the chamber is evacuated and shaken for 3 minutes. The extracted gases are ejected without loss of solution as described by Van Slyke ((12) p. 240). 1 drop of the sodium hyposulfite solution *without anthrahydroquinone sulfonate catalyst* is added to the blood and the extraction *in vacuo* is then repeated, this time for 2 minutes. After this treatment, there is no measurable amount of oxygen left in the blood. After

<sup>2</sup> When analyzing for  $O_2 + CO$ , the stop-cock is turned at this point, a little of the hyposulfite solution is run up into the capillary leading to the cup of the chamber, the stop-cock is sealed with mercury, and a reading  $p_o'$  is taken with the *mercury* meniscus at the 50 cc. mark. The hyposulfite is then ejected.

each extraction the ejection of the gas from the chamber must be complete. If a drop of blood follows the ejected bubble of gas up into the cup its loss is of no importance. The drop of blood which is exposed to air in the cup is not returned to the chamber, but is removed from the cup before the stop-cock is sealed with mercury for the next extraction.

*Absorption of Carbon Monoxide by Reduced Blood*—The de-oxygenated gas from the attached container is admitted into the chamber over the reduced blood. It is followed by a little mercury, to fill the connecting tubes, and to seal the stop-cock of the chamber. After this the attached container is disconnected.

The leveling bulb of the chamber is placed in a position slightly lower than the bottom of the chamber, and the stop-cock between the two is opened. *The water jacket of the manometric chamber is completely covered with tin foil to exclude all light* (the tin foil may be fastened in place with adhesive tape). The chamber is then slowly shaken for 30 minutes. The speed of shaking should be such that the blood, floating on the 10 or 15 cc. of mercury in the chamber, is whirled about the wall but is not broken up into foam. After equilibration, the tin foil is removed and the unabsorbed gas is ejected without any loss of blood. 4 drops of caprylic alcohol are added to the reduced blood in the chamber, and the stop-cock of the chamber is sealed with mercury.

*Determination of CO Absorbed by Blood*—Through a mercury seal ((8) p. 126) 13 cc. of the air-free ferricyanide reagent from the oil-protected burette are admitted into the chamber with the blood. Before the acid ferricyanide is admitted the chamber is partly evacuated, so that the surface of the blood is in the broad part of the chamber. After each 2 cc. admitted, the stop-cock of the chamber is closed, and while the burette is held firmly in place, the chamber is shaken gently two or three times to avoid the formation of large masses of protein precipitate which would be likely to adhere to the glass walls.

After the addition of the ferricyanide reagent, the stop-cock of the chamber is sealed with mercury, and the mercury in the chamber is lowered to or slightly below the 50 cc. mark, so that when the chamber is shaken mercury will not be thrown about its walls, with reduction of the ferricyanide. The evacuated chamber is shaken for 7 minutes to extract the CO from the large volume of solution.

From this point, the procedure for the determination of the CO absorbed by the blood is that described by Sendroy and Liu. The following additions to the technique are matters of convenience.

Before the Hempel pipette is used, some of the pyrogallate solution, together with any blood coagulum which may be present from a preceding analysis is run out to clear the stop-cock *a* (Fig. 1 of Sendroy and Liu (6)) of any air that may be present. Then a little caprylic alcohol is put into cup *c* and a drop is admitted into the capillary *r*. The caprylic alcohol prevents foam formation in the pyrogallate, and increases the ease with which the bubble of  $N_2 + CO$  gas can be returned to the chamber. In using the Hempel pipette, it is convenient to support its weight by a light wire or chain.

After removal of the blood solution from the chamber, the latter may be easily cleaned by the use of small portions of alcoholic KOH and alkaline hyposulfite. When the gas is returned to the chamber from the Hempel pipette, it is followed by the drop of caprylic alcohol. During the absorption of CO by cuprous chloride, some of the absorbent may precipitate on the walls of the chamber. After the analysis is finished the precipitate is readily dissolved by a mixture of saturated NaCl and 1 N  $H_2SO_4$ .

### *Calculation*

The pressure at 50 cc. of the sample, is calculated as

$$(1) \quad P_S = p_1 - p_0$$

The CO pressure at 0.5 cc. volume is calculated as

$$(2) \quad P_{CO} = p_2 - p_1 - c$$

$$(3) \quad \text{Per cent CO} = \frac{f_1 P_{CO}}{f_2 P_S}$$

$$= \frac{100 \times \text{cc. CO in sample}}{\text{cc. volume of sample}}$$

$p_1$  and  $p_2$  are the readings taken before and after the absorption of CO with Winkler's cuprous chloride solution in the Sendroy-Liu blood analysis.

$f_1$  is the factor from Table I, corresponding to the temperature and volume (0.5 cc.) at which  $P_{CO}$  is determined.

## VI. Manometric CO by Absorption

TABLE I

*Factor  $f_1$  by Which  $P_{CO}$ , Measured at 0.5 Cc. Mark, Is Multiplied to Give 100 Times the Volume, in Cc. at 0°, 760 Mm., of Carbon Monoxide Present in Gas Sample Analyzed*

Temperature °C.	Factor $f_1$
10	0.0687
11	85
12	82
13	79
14	77
15	74
16	71
17	69
18	67
19	64
20	62
21	60
22	57
23	55
24	52
25	49
26	47
27	45
28	43
29	40
30	38
31	35
32	33
33	31
34	29

$f_2$  is the factor from Table II, corresponding to the temperature and volume (50 cc.) at which  $P_S$  is determined.<sup>3</sup>

<sup>3</sup> The calculation for the amount of  $O_2 + CO_2$  present, when the readings  $p_1'$  and  $p_0'$  are taken,<sup>1,2</sup> is performed as follows: From the  $p_1'$  and  $p_0'$  readings taken with the gas at 47 cc. volume, the pressure at 50 cc., of the  $N_2 + CO$  left after hyposulfite treatment is given by the equation

$$(4) \quad P_{N_2 + CO} = 0.94 (p_1' - p_0')$$

(See discussion of calculation by Van Slyke and Sendroy (7).) The content

TABLE II

*Factor  $f_2$  by Which  $P_S$ , Measured at 50 Cc. Mark, Is Multiplied to Calculate the Volume in Cc. of Gas Sample Reduced to 0°, 760 Mm.*

Temperature °C.	Factor $f_2$
10	0.0634
11	31
12	29
13	27
14	24
15	22
16	20
17	18
18	15
19	13
20	11
21	09
22	07
23	05
24	02
25	00
26	0.0598
27	96
28	94
29	92
30	90
31	88
32	86
33	84
34	82

of  $N_2 + CO$  is given by the equation

$$(5) \quad \text{Per cent } N_2 + CO : \frac{100 P_{N_2 + CO}}{P_S}$$

from which the oxygen, and the  $CO_2$ , which is negligible in ordinary atmospheric air, are calculated as

$$(6) \quad \text{Per cent } O_2 + CO_2 = 100 - \text{per cent } (N_2 + CO)$$

In atmospheric air the  $CO_2$  is usually negligible in comparison with the  $O_2$ , so that Equation 6 gives the  $O_2$  content.

The factors of Table I are calculated by Equation 4 of Van Slyke and Neill (10), which gives the cc. of CO reduced to 0°, 760 mm., in the blood sample analyzed. The factors thus obtained are multiplied by 1.067 to give the cc. of CO in the gas sample, since only 0.937 or  $\frac{1}{1.067}$  of the CO in the gas is absorbed by the blood. The factors obtained are then multiplied by 100, in order to give at once results in volumes per cent. The complete calculation of the factors is therefore expressed by the equation:

$$\text{Factor } (f_1) = \frac{a}{760 (1 + 0.00384 t)} \left( 1 + \frac{S}{A - S} \alpha' \right) \times 106.7$$

$t$  is the temperature in degrees centigrade;  $a$  is the volume, 0.5 cc., at which the pressure  $P_{CO}$  is measured;  $S$  is the volume of solution, 18 cc., present in the chamber when the CO is extracted from the blood-ferricyanide mixture;  $A$  is the capacity of the chamber, 50 cc.;  $\alpha'$  is the distribution coefficient of CO between gas and water phases, as shown in Table I of Van Slyke and Neill.

The factors of Table II are calculated from the equation:

$$\text{Factor } (f_2) = \frac{a}{760 (1 + 0.00384 t)} \frac{0.0658}{1 + 0.00384 t}$$

when  $a = 50$  cc.

The  $c$  correction is determined by repeating the procedure described above in all respects, except that the steps involved in handling the air sample are omitted. The blood is reduced, the air-free ferricyanide reagent is at once added, and the determination from this point is finished as above. Two readings are made at the 0.5 cc. mark,  $p_1$  and  $p_2$  (corresponding to readings  $p_2$  and  $p_3$  of the air analysis) before and after the addition of Winkler's solution to absorb CO. The  $c$  correction is calculated as

$$p_1 - p_2 = c$$

The  $c$  correction is the sum of two components. One is the  $c_2$  correction of the Sendroy-Liu method ((6) p. 139). The other is a correction for a slight amount of CO (0.08 to 0.18 volume per cent) which, in agreement with the work of Nicloux (3) and

McIntosh (4), has been found to be apparently present in normal blood. Because this CO content of the blood is variable, the *c* correction must be determined with a reduced portion of the same blood that is used for the air analysis.

*Correction Factor 1.067*—The reaction,  $\text{Hb} + \text{CO} \rightleftharpoons \text{HbCO}$  is a reversible one. Since the final result of shaking the  $\text{N}_2 + \text{CO}$  mixture with the blood must be an equilibrium between the CO in the gas phase and the CO bound to hemoglobin in the liquid phase, it is theoretically impossible that all of the CO present should be combined with hemoglobin. There is not much known about the system: reduced hemoglobin, CO hemoglobin, and CO (13). However, since there is a variation in the blood of different species, in the oxygen dissociation curves, and in the relative affinity constant of hemoglobin with respect to  $\text{O}_2$  and CO, it is not unreasonable to expect that there may also be a similar species specificity with respect to the CO dissociation curves. This may or may not be a factor affecting the distribution of CO between the gas phase and the reduced blood after the 30 minute period of shaking required for the present technique of analysis. However, under the conditions outlined above for the absorption of CO by reduced blood, *when ox blood is used*,  $93.7 \pm 2.4$  per cent of known amounts of CO (in concentrations of 0.05 to 0.30 per cent) in air have been recovered from the reduced blood used for the analysis. The corresponding correction factor 1.067 has been found constant to within  $\pm 2.4$  per cent, as is shown in Tables III and IV, and is accordingly used in calculating the factors of Table I.

Arnold *et al.* (1) obtained complete recovery of CO in air exposed to reduced blood. However, they did not control the CO possibly originally present in the blood they used. McIntosh (4), using a Harington (14) modification of the Van Slyke apparatus, controlled the CO content of the human blood used. He assumed that his analytical procedure sufficed "for nearly complete absorption of the carbon monoxide." Nicloux (5) on the other hand, although pointing out the existence of CO in normal blood (3), apparently made no correction for it. Without this control, his eudometric analyses of three gas mixtures ranging from 0.24 to 0.45 per cent of CO in air indicate an absorption of about 93.3 per cent of the CO by beef blood.

## EXPERIMENTAL

In establishing the order of accuracy and the constancy of the results obtained by the technique of analysis outlined above, two sets of experiments were performed.

In the first set, the results of which are given in Table III, CO gas was diluted to a measured extent with CO-free air. The purity of the CO gas was controlled by analyses for CO<sub>2</sub> and O<sub>2</sub> (7, 11) and by ascertaining that the gas could be completely absorbed by Winkler's cuprous chloride solution. Several lots of pure CO were made from formic and sulfuric acid. In none of these preparations was the gas found to be less than 98 per cent pure.

The CO was run into the chamber of the manometric apparatus over mercury, and the pressure (150 to 200 mm.) was measured at 2.0 cc. volume according to the technique (7) used for the measurement of gas samples. For these measurements the gas volume held above the 2 cc. mark was determined by calibrating the chamber with mercury (7), since the volume above a mercury meniscus is greater than above a water meniscus at the same mark. The volume of the CO at 0°, 760 mm., was calculated by multiplying the pressure of the sample at the 2.0 cc. mark by the factor

$$\frac{a}{760(1 + 0.00384t)}$$
 where  $a$  is the volume of gas held by the chamber over a mercury meniscus at the 2 cc. mark. After the measurement, the gas was completely run over into a large calibrated gas sampling tube of a size to dilute the CO to the desired volume. With negative pressure in the sampling tube, air was admitted until the mixture was just at atmospheric pressure. The sampling tube was then placed in a water bath slightly below room temperature, and again the contents were equilibrated with the atmosphere. The air used was also analyzed for CO by control analyses. At no time was there demonstrable a significant amount of CO in the air used for dilution.

In the second set of experiments, the results of which are recorded in Table IV, the CO mixed with air was measured in a different manner. Fresh ox blood was treated to give it a CO content of 8 to 10 volumes per cent, and the exact CO content was found by analysis by the Sendroy-Liu method. A sample of 1

cc. of the blood was then treated with acidified ferricyanide in the manometric chamber and the liberated CO was extracted. The CO gas thus obtained was diluted by the admission into the chamber, of 45 to 47 cc. of room air. The CO + air mixture

TABLE III  
*Results of Analyses of Air to Which Measured Amounts of CO Were Added Directly*

Sample No.	CO per 100 cc. air		Ratio (a) (b)	Deviation from average ratio 0.937
	Regained from blood (a)	Present (b)		
	cc.	cc.		
1	0.087	0.094	0.925	-0.012
	0.087		0.925	-0.012
	0.086		0.915	-0.022
	0.091		0.968	+0.031
	0.085		0.904	-0.033
2	0.047	0.049	0.959	+0.022
	0.045		0.919	-0.018
	0.047		0.959	+0.022
3	0.181	0.200	0.906	-0.031
	0.181		0.906	-0.031
	0.180		0.900	-0.037
	0.182		0.911	-0.025
4	0.184	0.197	0.920	-0.017
	0.179		0.898	-0.039
5	0.180	0.200	0.900	-0.037
6	0.200	0.209	0.957	+0.020
	0.192		0.919	-0.018
	0.196		0.938	+0.001
	0.185		0.886	-0.051
	0.190		0.910	-0.027

was transferred through the capillary side arm into the gas sample container. After the chamber had been thoroughly cleaned the gas mixture was analyzed according to the procedure given above.

The results of Tables III and IV show that the values for the

ratio  $\frac{a}{b}$  ( $= \frac{\text{CO found}}{\text{CO present}}$ ) by the first set of analyses of six different gas mixtures average 3 per cent lower than those found for the second set of analyses of eight gas mixtures. Since there is no reason to doubt the validity of either set of results, equal weight has been given to both, and an average value of 0.937 for the ratio

TABLE IV  
*Results of Analyses of Air to Which Were Added Portions of CO Released from Analyzed Blood*

Sample No.	Approximate concentration of CO in gas mixture <i>per cent</i>	CO in air sample		Ratio (a) (b)	Deviation from average ratio 0.937
		Regained (a) cc.	Present (b) cc.		
1	0.27	0.0856	0.0900	0.952	+0.015
	0.27	0.0847		0.942	+0.005
2	0.28	0.0883	0.0994	0.935	-0.002
3	0.27	0.0834	0.0853	0.978	+0.041
4	0.26	0.0779	0.0807	0.965	+0.028
5	0.30	0.0941	0.1020	0.922	-0.015
	0.31	0.0871	0.1020	0.951	+0.014
6	0.29	0.0909	0.0975	0.932	-0.005
7	0.27	0.0826	0.0847	0.976	+0.039
8	0.27	0.0853	0.0870	0.981	+0.044

$\frac{a}{b}$  has been derived. The reciprocal of this is the factor 1.067 used in calculating the factors of Table I, to give the actual amount of CO present in analyzed gas mixtures.

#### SUMMARY

A method is described, whereby air containing carbon monoxide in concentrations from 0.05 to 0.3 per cent may conveniently be analyzed in the Van Slyke-Neill apparatus. The CO is

first combined, in the chamber of the apparatus, with the hemoglobin of completely reduced blood, and the CO content of the blood is then determined by the method of Sendroy and Liu.

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## A METHOD FOR THE MICRO GRAVIMETRIC DETERMINATION OF SILICA IN TISSUE

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Gravimetric procedures as ordinarily performed are not sufficiently sensitive for the reliable determination of amounts of silica less than 5 mg. in weight. With the volumetric procedure (7), which is based on the titration of silica by an acid in the presence of fluoride ( $\text{SiO}_2 + 6\text{KF} + 4\text{HCl} \rightarrow \text{K}_2\text{SiF}_6 + 2\text{H}_2\text{O} + 4\text{KCl}$ ) differences of 0.1 mg. of silica are difficult to detect and the results are affected by the presence of salts other than those of the alkali metals and ammonia. The colorimetric determination (1) of silica has been investigated in this laboratory (4). It is sensitive to 0.01 mg. and is quickly and easily applied. But as these methods have to be standardized by gravimetric means, less confidence is apt to be placed in their less direct results. Therefore, it was felt that the gravimetric principle would furnish a more reliable and fundamental basis for a micro method, if such could be worked out. This paper gives the results of an attempt to develop such a micro gravimetric method.

The micro gravimetric procedure evolved is based on the same chemical principles as the usual gravimetric analysis for silica, but the technique is entirely different. In working out the method use was made of the apparatus and methods described by Emich (2) and by Pregl (6). A micro balance<sup>1</sup> is essential for all micro gravimetric work.

Special precautions must be taken when attempting to perform quantitative operations on a small scale. That the material to be sampled be strictly uniform was particularly important. The

<sup>1</sup> The type of micro balance manufactured by Kuhlmann (Hamburg) or Becker (New York) is suitable.

vessels used were as small as was practicable. Crucibles of 10 cc. capacity were employed, as this size was just sufficient to permit all the necessary operations being performed therein. Since the transference of the material from one vessel to another is always a potential source of error, the silica was not removed from the crucible from the time the sample was weighed out until the analysis was completed. This was made possible by the "*Stäbchen*" method of filtering to be described later. The crucibles were handled with platinum-tipped tongs when heating and with platinum-tipped forceps during all other operations. Care was taken to keep dust from entering the crucibles. Save when actually being heated, etc., they were kept in pairs in 250 cc. Pyrex beakers covered with watch-glasses. These beakers were placed in a desiccator between operations. A dust shield consisting of a small sheet of stainless steel was placed above the heating devices. Another essential for obtaining consistent results is to add exactly the same amounts of reagents every time and to heat the crucibles for the same length of time during each ignition. Careful attention to detail is imperative for the success of the method.

The crucibles were cleaned with chromic acid (8) or by boiling a little perchloric acid in them. After cleaning and rinsing, the crucibles were ignited in the flame of a Bunsen burner, allowed to cool in the desiccator for half an hour, and weighed. Unless it was desired to know the weight of the residue after ashing, the crucibles were weighed only to the nearest 0.1 mg., which was as close as the next weighing could be performed since the tissue was slightly hygroscopic. Samples of the dry, powdered tissue ranging from 100 to 500 mg. were added to the crucibles and weighed quickly to the nearest 0.1 mg. For each crucible there was a counterpoise (6), which was conveniently handled with forceps whose tips were covered with fine rubber tubing.

The samples of tissue were ashed to remove organic matter. A hot plate covered with asbestos paper was preferred for starting the ashing, *i.e.* until the mass was completely charred and volatile matter driven off, because the temperature could be kept more easily under control. Various types of muffles were tried for completing the ashing, but finally it was found that the operation could be performed most readily and quickly in the ring burners used for the fuming operations to be described below. The initial

stages of the ashing must be performed with great care as the charring mass tends to rise up and over the edge of the crucible, and occasionally to take fire. After increasing the temperature until the crucible had been at red heat for sufficient time to burn off most of the carbon, it was held with the tongs so that the full blast of the flame played on the bottom for about half a minute. If any specks of carbon remained then, they were ignored as they were removed in subsequent ignitions. The crucible now contained the silica, together with other non-combustible substances. If the original material contained mineral silicates, fusion with sodium carbonate<sup>2</sup> would be necessary at this juncture; such was not the case with the material under consideration.

At first it was thought that it might be suitable to weigh the sample, remove everything but the silica, and weigh the residue as  $\text{SiO}_2$ . For these operations porcelain crucibles would serve. However, this procedure did not give satisfactory results. Whenever platinum was employed for this purpose the weight of the crucibles was affected so much during the ashing operation that it was impossible to state the weight of the residue to within 0.01 mg. It was therefore decided that it would be necessary to revert to the common practice of volatilizing the silica with hydrofluoric acid and determining the silica from the loss in weight on volatilization. This, of course, entailed the use of platinum crucibles.

Following the suggestion of Kraut (5), an attempt was made to ash the tissue and treat the ash directly with the hydrofluoric acid without separating the silica from the other substances. It was found, however, that on adding the acids the salts formed showed a marked tendency to creep up the sides of the crucible and over the edge, whatever the mode of heating. Therefore, the procedure adopted was to ash the tissue, render the silica insoluble, filter off the other substances, and treat the purified silica with hydrofluoric acid.

The method of rendering silica insoluble is simply to heat it in acid solution, which at the same time dissolves practically all other material present. The acid commonly employed is hydrochloric.

<sup>2</sup> Potassium carbonate should not be used if perchloric acid is to be employed in the subsequent treatment, since potassium perchlorate is among the least soluble of the salts of perchloric acid.

However, it possesses certain disadvantages (3), among which are its solvent action on silica and the trouble incurred by the fact that the solution has to be evaporated to dryness and heated in an oven for some time. A more satisfactory agent was found by Willard and Cake (9) in the form of the dihydrate of perchloric acid, which is a powerful dehydrating agent at its boiling point, 203°. By boiling the silica with this acid for 15 minutes the dehydration is completed, and the mixture does not require to be taken to dryness. Thus the separation is accomplished in but a fraction of the time required with hydrochloric acid. Other advantages attending the use of perchloric acid are that less silica is lost in the filtrate and that the perchlorates as a class are more soluble than the corresponding chlorides. This makes the

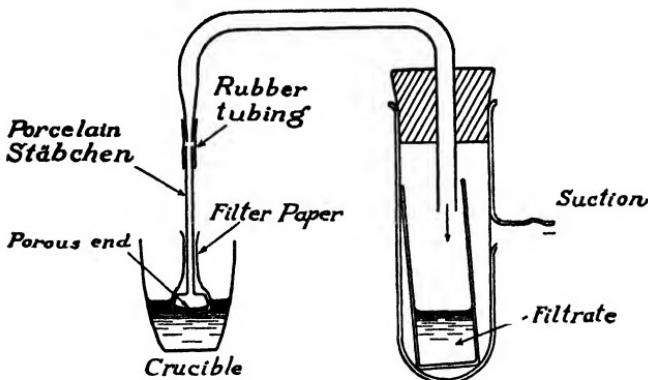


FIG. 1. Method of filtering by *Filterstäbchen*

separation more complete and easier to perform. The results obtained with this dehydrating agent were more consistent than those obtained when hydrochloric acid was used.

The operation of dehydrating the silica with perchloric acid was performed as follows: To the white residue in the crucibles were added 3 cc. of 70 per cent perchloric acid. The crucibles were covered and placed on a hot plate covered with thin asbestos paper in a fume cupboard with a strong draught. They were boiled for 10 to 15 minutes, but not allowed to go dry. Then they were set on a piece of asbestos to cool.

When cool, the condensate on the under side of the covers was washed off into the crucibles, the latter were almost filled with

silica-free distilled water, and the solution was filtered off. The transference of the precipitate from the crucible was avoided by the method of filtering evolved by Emich (2), which consists in sucking off the solution through a *Filterstäbchen*. The manner in which it was used is illustrated in Fig. 1.

In Emich's methods the *Stäbchen* is left in the vessel after the filtration, the whole being dried and weighed together, but in the case of silica determinations this was impossible as it was desired to fume the residue with hydrofluoric acid, which would attack the porcelain *Stäbchen*. To overcome this difficulty a small disc of fine pore, low ash filter paper (Whatman's No. 42, 5.5 cm.) was moistened and placed over the end of the *Stäbchen* with the edges folded down neatly around the stem and held by a spring clothespin. It was set on top of a drying oven. When dry the paper retained its shape on removing the clip. The filtration was carried out as follows: A *Stäbchen* was moistened at the upper end, inserted in the rubber tubing of the holder, and the filter paper moistened before filtration was commenced. By means of forceps the crucible was held up around the *Stäbchen* to which suction was applied, as shown in Fig. 1, and the soluble salts filtered off. The crucible and filter paper were thoroughly washed with distilled water<sup>3</sup> and the filter paper loosened with the forceps and shoved down into the crucible. The latter was then set in a drying oven at 110°, while the contents of the next crucible were filtered. At first the filtrates were all examined for silica, but after it was found that they contained a negligible amount, they were discarded without examination during later analyses.

After the filter papers were quite dry, they were burned off by heating the crucibles in the ring burners, cautiously at first, to avoid igniting the filter papers. Then the crucibles were set in their beakers to cool.

This left all the silica in the crucibles in a fairly pure state. Its weight was determined by fuming, first with sulfuric acid, and then with hydrofluoric acid plus sulfuric acid, the crucible being weighed after each operation. At first the fuming was performed on the hot plate. This means of heating had several drawbacks,

<sup>3</sup> It is necessary to wash the filter paper at least five or six times, as the presence of any perchlorates may cause an explosion during the next ignition.

among which were the relatively low maximum heat, the difficulty of controlling the temperature, and the tendency of the residue to creep up the side of the crucible. It was thought that the application of heat from above would obviate these difficulties. This could be done uniformly by means of flames radiating horizontally inward from a ring surrounding the crucible. Suitable ring burners were not to be found on the market. Accordingly, a number of ring burners were manufactured in the department. They gave a blue flame which could be regulated from the merest glow to a length of one inch, as shown in the sectional diagram (Fig. 2).

Each crucible received 0.10 cc. of the purest concentrated sulfuric acid obtainable, and was then placed in the ring burner so that the flames came just below the rim of the crucible. Sufficient heat was applied to make the edge of the crucible dull red. When

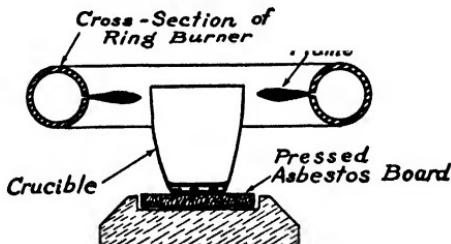


FIG. 2. Cross-section of ring burner with crucible

fumes ceased, the flames were turned on full, the time noted, the crucible grasped by one side with the tongs and raised so that it was completely ignited and then held on a slant so that the bottom was as hot as possible, to complete the ignition of the residue in the bottom. 20 or 30 seconds after noting the time, the crucible was removed from the flame and immediately placed in its beaker in the desiccator. After cooling for 30 minutes the crucibles were carefully wiped with clean, dry chamois and set in a row in the balance case. They were then weighed to within 0.001 mg., notwithstanding the fact that a great deal of confidence cannot be placed in the last figure when weighing 8 gm. crucibles, owing to the influence of temperature and humidity changes. Then the crucibles were placed in a duplicate set of beakers reserved for this operation, and 0.50 cc. of hydrofluoric acid (as low as possible in

non-volatile matter) was added to each crucible with a Bakelite pipette. Then 0.10 cc. of the sulfuric acid was added, and the crucibles fumed off as before, but the heat was applied very cau-

TABLE I  
*Typical Results with Micro Gravimetric Method*

Material	Sample	HF loss	Blank	SiO <sub>2</sub> found	SiO <sub>2</sub> in sample
	mg.	mg.	mg.	mg.	per cent
Silicic acid	2.00	1.801	+0.040	1.841	92.0
	7.62	6.901	+0.040	6.941	91.1
	16.88	15.144	+0.040	15.184	89.9
Average.....					91.0
Cow Lung B10	611.6	0.489	+0.116	0.605	0.099
	620.1	0.553	+0.116	0.669	0.108
Average.....					0.103
Cow Lung B10	574.0	9.335	+0.116	9.451	100.7*
+ silicic acid	9.67				
"Drosil"† silicic acid-containing tablets	438.14	24.724	+0.010	24.734	5.65
	549.95	31.875	+0.010	31.885	5.79
Rabbit Lung 365	274.1	0.146	-0.011	0.135	0.049
	364.0	0.163	-0.011	0.152	0.042
"      " 380	398.6	0.044	-0.006	0.038	0.010
	418.9	0.033	-0.006	0.027	0.007
"      " 656	283.7	0.741	-0.027	0.714	0.252
	257.3	0.682	-0.027	0.655	0.254
"      " Liver 656	515.5	0.083	-0.047	0.036	0.007
	486.3	0.067	-0.047	0.020	0.004
"      " Kidney 656	91.0	0.032	-0.024	0.008	0.009
	122.4	0.031	-0.024	0.007	0.006
Silicotic lung	228.0	7.789	-0.028	7.761	3.40
	185.1	6.259	-0.028	6.231	3.37
"      "	295.5	22.731	-0.028	22.703	7.68
	314.4	23.377	-0.028	23.349	7.42

\* Silica recovered 8.86 mg. = 100.7 per cent.

† A European pharmaceutical preparation containing colloidal silica, extract of drosera, and saccharin.

tiously this time as the liquid had a tendency to splutter. As soon as bubbles ceased to form, and the bulk of the liquid had gone down considerably, the heat was increased until almost dull

red. A few trials showed when it was safe to increase the heat. The remainder of the operation was carried out as before, and the crucibles weighed with the same accuracy. In some cases the fuming with hydrofluoric acid was repeated to insure that all the silica had been driven off. However, there was seldom any left after the first operation. A blank, consisting of the same amounts of reagents used in the determination, was run frequently, and its result deducted (if a loss)<sup>4</sup> from the loss in weight on fuming.

A few typical results are given in Table I. The time required to make duplicate determinations on two tissues (four crucibles) was about 8 hours.

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<sup>4</sup> With the sulfuric acid and hydrofluoric acid first used the blank value determined was always positive, but with the introduction of a new brand of sulfuric acid and hydrofluoric acid which were very low in non-volatile matter, the blank was found to be negative. This negative value was probably due to the loss in weight of the platinum crucibles during the heating process.

## STUDIES ON BLOOD GLYCOLYSIS

### I. EFFECT OF ARSENATE

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Meyerhof (8) has shown that arsenate stimulates the decomposition of carbohydrate and of hexosephosphate esters by the glycolytic enzyme of muscles. This observation brings muscle glycolysis into close relationship with fermentation of sugar by yeast or yeast products. Harden and Young (6) who discovered the stimulating action of the phosphate ion on the process of alcoholic fermentation were led to investigate the effect of the related arsenate ion and found that this exerted a similar stimulating action. Neuberg and Kobel (10) later demonstrated that vanadate likewise stimulates yeast fermentation, a fact which Braunstein (3) has recently shown to hold also for blood glycolysis.

In the course of our studies on the effect of isotonic solutions of various salts on glycolysis of blood we found that NaCl, NaI, or NaBr either had no effect or caused only a slight decrease in the glycolytic activity, whereas an isotonic arsenate solution suppressed glycolysis almost as completely as does NaF. Inasmuch as we diluted blood with an equal volume of the salt solution, it means that  $M/16$  arsenate can bring about almost total cessation of glycolysis. Prompted by this observation we extended the investigation to various concentrations of arsenate, studying the changes in sugar, lactic acid, and inorganic phosphate by a procedure discussed elsewhere (9) and found that the arsenate produced a definite though small inhibition of glycolysis even in a concentration of  $M/5120$ .

The results of these experiments are recorded graphically in Figs. 1 and 2 which show the effect of different arsenate concentrations, made up in physiological saline, on the disappearance of

## Blood Glycolysis. I

sugar and formation of lactic acid and inorganic phosphate in glycolyzing dog blood. From these typical experiments it is clear that even  $M/1280$  sodium arsenate solution causes an unmistakable inhibition of the glycolytic process, which becomes progressively more marked as the arsenate concentration increases. The results in Fig. 1 represent the effect of 4 hours incubation on the glycolysis, while in Fig. 2 are recorded the results of experiments with

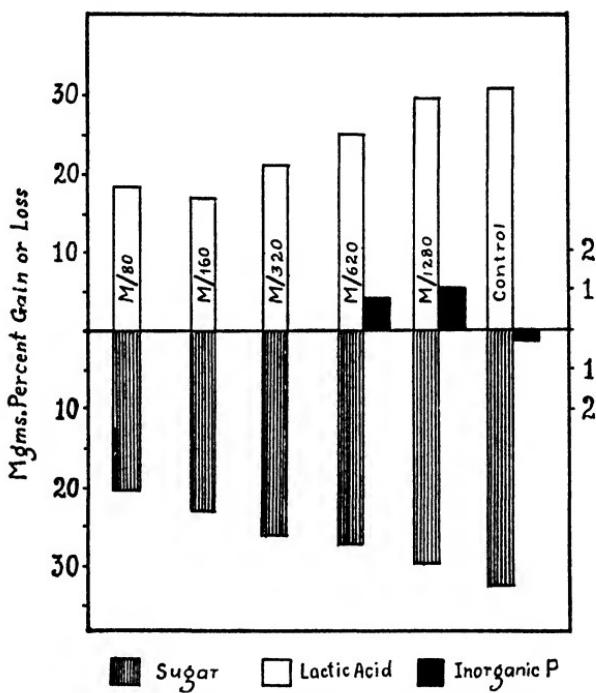


FIG. 1. Effect of various concentrations of arsenate on glycolysis of dog blood.

smaller arsenate concentrations, ranging from  $M/1280$  to  $M/5120$ , and for a period of 2 and 4 hours of incubation. In this case the effect does not become apparent until after 4 hours of incubation. It will be noted that, while the glycolysis, *i.e.* disappearance of sugar and the formation of lactic acid, is inhibited, there is simultaneously a more or less extensive liberation of inorganic phosphate which seems to be progressive during the glycolysis experi-

ment. In our original experiments we were unable to determine the phosphate with any certainty in the presence of much arsenate, and our determinations were limited to those with the lowest arsenate concentrations. Nevertheless it was clear that an increase in inorganic phosphate was an invariable accompaniment of glycolysis in the presence of arsenate.

Since the above experiments were performed Barrenscheen and Hübner (2) reported that arsenate has a marked stimulating effect on glycolysis, and later Braunstein (3) also showed that the

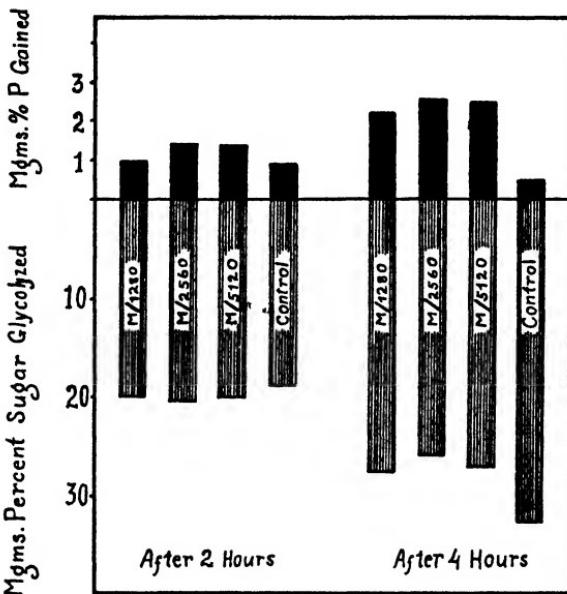


FIG. 2. Effect of various concentrations of arsenate on glycolysis of dog blood. The shaded boxes represent sugar, the solid boxes, inorganic P.

stimulation of the glycolysis was associated with an extensive liberation of inorganic phosphate. The fact that these reports corroborated our own findings so far as the changes in phosphates are concerned, but contradicted our results with regard to the rate of the disappearance of sugar, led us to reexamine the question with a view to finding the possible causes of such a discrepancy.

Two conditions which were different in our own experiments and those performed by Barrenscheen or Braunstein commanded

attention; namely, that we dissolved the arsenate in physiological saline while the other investigators used Locke's solution instead, and secondly, that we worked with dog blood while the others used rabbit blood. The glycolytic behavior of dog and rabbit blood does, of course, present certain distinct differences, as can be seen from Fig. 3 in which typical glycolysis curves are shown of dog and rabbit blood incubated at 37° for different lengths of time.

We performed, therefore, new experiments, using both dog and rabbit blood and dissolving the arsenate either in physiological

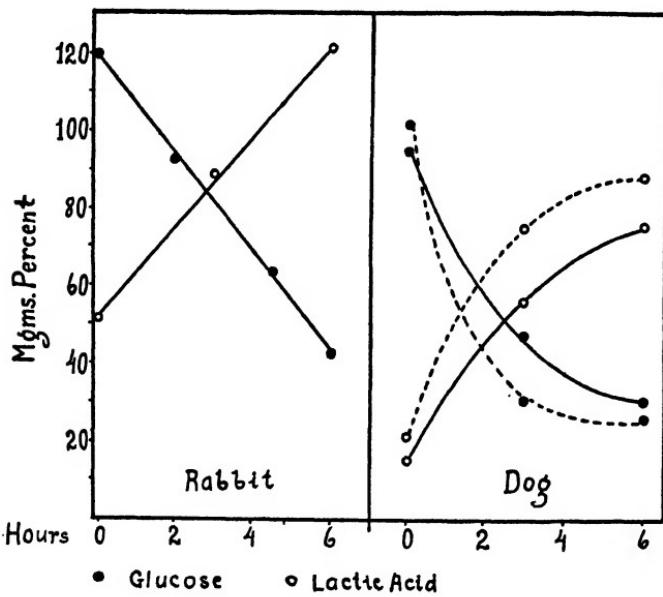


FIG. 3. Blood glycolysis

saline or in Locke's solution. The results of these experiments are recorded graphically in Figs. 4 and 5. We also developed the phosphate determination so that it could be made in the presence of various concentrations of arsenate.

These experiments show strikingly the effect of arsenate on the hydrolysis of organic phosphates. Engelhardt and Braunstein (4) have also reported a similar observation. Our experiments show the relationship between the arsenate concentration and the liberation of phosphate. The progressive rise in inorganic phos-

phate, which we observed in glycolyzing dog blood, was accompanied by a gradually increasing suppression of glycolysis with increasing arsenate concentration. In this respect the experiments present nothing unusual, because it is now generally recognized by students of this problem that any factor which inhibits or checks

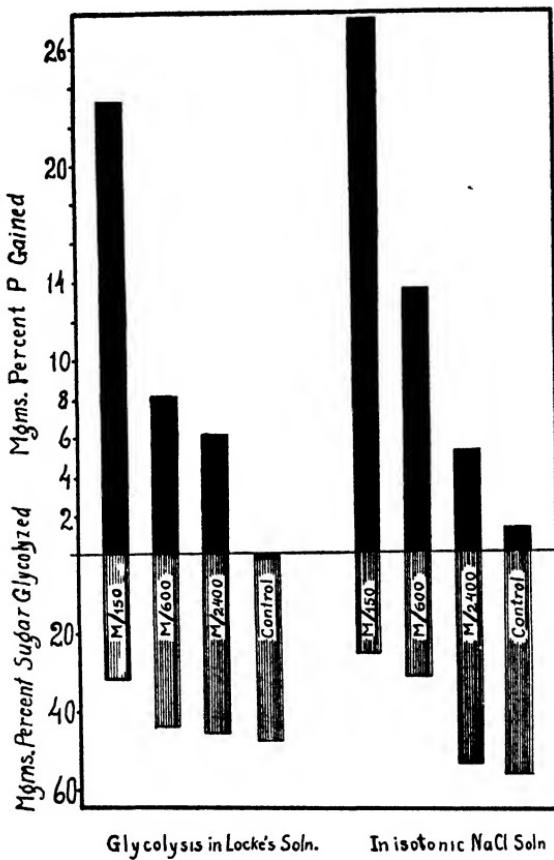


FIG. 4. Effect of arsenate on dog blood. The shaded boxes represent sugar; the solid boxes, inorganic P.

the glycolytic process conduces at the same time to the liberation of phosphoric acid from its esters (*cf.* Rona and Iwasaki (14) on the effect of checking glycolysis by the removal of sugar; Engelhardt and Braunstein (4) by fluoride; Engelhardt and Ljubimowa (5) by

hemolysis; Barrenscheen and Braun (1) by various agencies). In fact, when glycolysis is checked by removing sugar and especially by cytolyzing the cells, there is actually an explosive liberation of inorganic phosphate. But the results of Engelhardt and Braun-

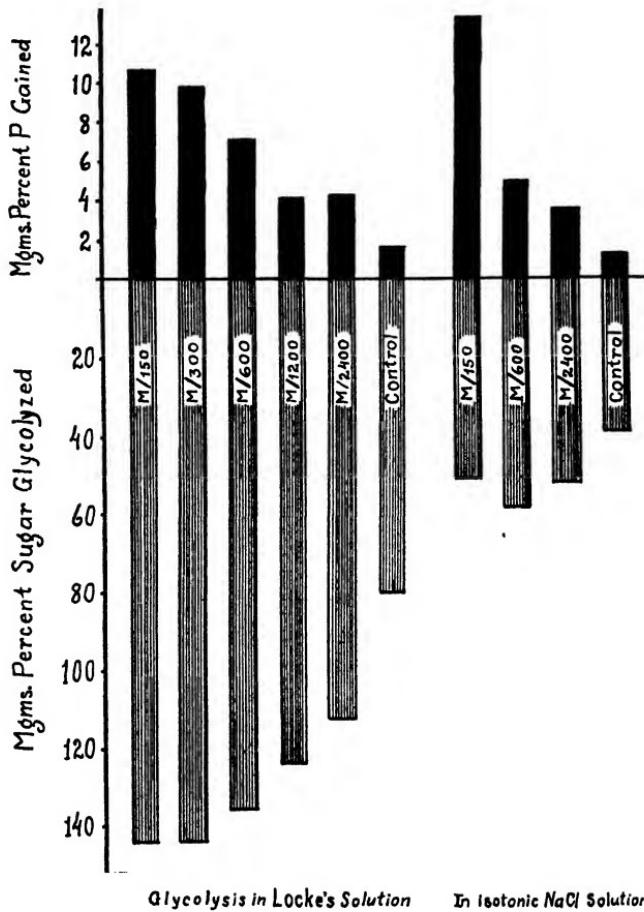


FIG. 5. Effect of arsenate on rabbit blood. The shaded boxes represent sugar; the solid boxes, inorganic P.

stein (4) and of the subsequent experiments of Braunstein (3) are much more difficult to interpret, inasmuch as here the liberation of phosphoric acid is actually accompanied by an increase in glycolytic activity.

Braunstein (3) using the Fiske-Subbarow method for the phosphate determination depends upon rapid reading of the colors to avoid errors due to the presence of arsenate, the extra color development from arsenate presumably being a slower process. This, of course, is an uncertain and indeed a very uncritical procedure for quantitative estimations. Using the Kuttner method, we have worked out the correction for the arsenate, determining the latter as phosphate. Over a wide range of arsenate concentrations we found the equivalent phosphate values to be in a direct linear ratio. The proportionality of color development holds only for a limited range of colorimeter readings, and we adjusted the concentration of the unknown so that it would not deviate by more than 12.5 per cent from the standard (the readings being 17.5 to 22.5 mm. with the standard set at 20 mm.). After determining P according to total color development, the P value equivalent to the As concentration was deducted, and the difference represented, therefore, the true phosphate. This procedure checked up well with known mixtures.

The results of experiments with dog and rabbit blood in both Locke's or isotonic salt solution are recorded graphically in Figs. 4 and 5. It will be noted that there was invariably a marked increase in the inorganic phosphates, which becomes greater with the progressive rise in the arsenate concentration. It also seems that the increase in phosphate is greater in NaCl than in Locke's solution, this difference being perhaps more obvious in the glycolyzing dog blood than in the rabbit blood. The glycolysis of dog blood, judging by the amount of sugar disappearing, is definitely inhibited, the degree of inhibition varying with the arsenate concentration, as was also found in our earlier experiments. At the same time these experiments corroborate the findings of Engelhardt and Braunstein (4), Barrenscheen and Hübner (2), and of Braunstein (3), that arsenate greatly accelerates the glycolytic process in rabbit blood, this effect being more manifest in Locke's than in isotonic NaCl solution. In our experience, arsenate causes as great an increase in the inorganic phosphates of the glycolyzing system as does vanadate, the effect of which Braunstein considers rather extraordinary. It may also be observed that in the case of rabbit blood, both the liberation of phosphate and the glycolysis of sugar are much accelerated by arsenate. But in NaCl solution the

increase in inorganic phosphate is greater than in Locke's solution, while glycolysis is more accelerated in Locke's solution than in NaCl solution. We thus witness here a similar reciprocal relationship between the effect on the glycolytic process and on the hydrolysis of hexosephosphate esters (or perhaps the prevention of resynthesis of phosphate esters) as was already suggested by the experiments on dog blood.

Engelhardt and Braunstein (4) developed the hypothesis according to which the blood phosphate level during glycolysis is a balance between the  $H_3PO_4$  set free from easily hydrolyzable esters and its resynthesis to more stable esters. The increase in inorganic phosphate is, therefore, due to a failure of glycolysis, as can be demonstrated by such means as NaF which suppresses the glycolytic process. The experiments with arsenate somehow do not fit properly into this scheme. In this case, as was found by Engelhardt and Braunstein (4), Barrenscheen and Braun (1), and later by Braunstein (3) and also by us, the phosphate *invariably* increases, sometimes even to a very marked degree, in the presence of arsenate. Furthermore, in the case of rabbit blood this great increase takes place while the glycolytic process is also greatly stimulated. It is, of course, entirely conceivable, as Braunstein actually argues (3), that hexosephosphate esters may be replaced by hexosearsenate esters, which are perhaps more labile, and this would also account for the setting free of phosphoric acid. Attractive as such an interpretation may seem at first sight, it meets with certain very serious objections. The replacement of phosphate by arsenate seems to us highly improbable because the color produced by As is more than 3 times as intense as that produced by an equivalent amount of P. In the event of an exchange of free  $AsO_4$  and  $PO_4$  ions, one would naturally expect a gradual diminution in the total color evolution. Had such a thing actually happened, it could only have been interpreted as a diminution in the free phosphates. The analytical results, therefore, preclude any such assumption of an interchange of arsenate for phosphate in the hexose esters.

Braunstein furthermore suggests that the hexosearsenate esters might glycolyze more rapidly than hexosephosphates and thus account for the acceleration of the glycolytic process as is observed, for instance, in rabbit blood. This explanation, however, would

again fail to apply to dog blood, in which arsenate actually inhibits the glycolysis.

An examination of the results presented in Figs. 4 and 5 shows very clearly that arsenate has a definite effect, increasing the inorganic phosphates of the glycolyzing system proportionally to the arsenate concentration. Raymond (11) found that the phosphatase activity of yeast is markedly increased by 0.004 M arsenate. Unfortunately, this author, who also determined the phosphates by the Kuttner procedure, does not say whether he corrected for the color development due to the arsenate, which, of course, would materially affect the quantitative results. Macfarlane (7) found that arsenate stimulates phosphatase activity. One might thus be inclined to assume that the great liberation of phosphate noted in blood-glycolyzing systems in the presence of arsenate is likewise the outcome of the stimulation of the blood phosphatases. Braunstein denies the possibility of the phosphatase stimulation in blood. His contention is based on several arguments. First, he maintains that arsenate does not promote liberation of inorganic phosphate when glycolysis does not take place or is inhibited. In view of our experience with dog blood in which, in spite of the inhibition of the glycolysis, the inorganic phosphates do increase with rising arsenate concentration, this argument is deprived of its force. Roche and Roche (12) report that  $\text{CaCl}_2$  causes increased liberation of phosphate without affecting the glycolysis of the blood. If the glycolysis is checked by  $\text{NaF}$ , the addition of the  $\text{CaCl}_2$  within 30 minutes brings about reactivation of the glycolysis, but, if added after a lapse of 24 hours, there is no longer a reactivation of glycolysis but only an abundant liberation of phosphates. Obviously, therefore, the phosphatase and the glycolysis enzyme systems can be affected independently. The second argument, namely that in cytolized blood the addition of arsenate fails to affect the hydrolysis of phosphate esters and, therefore, cannot be effective in stimulating phosphatase activity, likewise has very little weight, inasmuch as in the hemolyzed blood the maximum liberation of phosphate has already taken place spontaneously. The third argument Braunstein bases upon experimental findings showing that the arsenate does not influence the blood phosphatase activity. Since the phosphatase activity is thus unaffected, Braunstein

argues that the liberation of phosphate in arsenate-containing glycolyzing systems must be due to a replacement of phosphate by arsenate esters. This last argument we tested by direct experimentation. As the source of phosphatase we used washed red blood cells which had been cytolized with an equal volume of distilled water. The technique of these experiments was essentially the same as recommended by Roche (13), the reaction being carried out at 37° at pH about 6.5 for 4 hours. A solution of magnesium hexosediphosphate served as a substrate, the equivalent of 3 mg. of esterified P being used in each test-tube. The experiments were properly controlled. The arsenate concentrations employed were 0.004, 0.002, and 0.001 M. Correction was made for the free phosphate in the hexosediphosphate solution, for phosphate liberated by the cytolized cells without any additional phosphate ester, as well as for the arsenate. The latter correction was made on the basis of direct determination on a corresponding arsenate concentration. The tests with arsenate have all shown an increased hydrolysis of the phosphate ester, as can be seen from the recorded data.

	Control	Arsenate		
		0.004M	0.002M	0.001M
P hydrolyzed, mg.....	0.242	0.503	0.405	0.357
" increase, mg.....		0.261	0.163	0.115
Increase, per cent.....		108	67.5	47.5

It is obvious, therefore, that arsenate does stimulate very markedly the phosphatase activity of the blood cells, this stimulation being directly related to the arsenate concentration, as was also the case in the blood glycolysis experiments.

We may conclude, therefore, that the great liberation of inorganic phosphate observed in blood-glycolyzing systems under the influence of arsenate is due to a stimulation of the blood phosphatases. The stimulation of the glycolyzing enzymes by arsenate, on the other hand, is not a general phenomenon. With rabbit blood this is very prominent, but with dog blood the arsenate acts as an inhibitor of glycolysis. Evidently the effect of arsenate on glycolysis depends upon the nature of the particular glycolyzing system. Just what part of this system is responsible for

either the stimulating or the inhibiting action of the arsenate must still wait for a solution.

#### SUMMARY

Arsenate may either accelerate or suppress blood glycolysis, the effect being more or less directly related to the concentration of the arsenate. Rabbit blood responds by increased glycolysis but dog blood responds by a diminished glycolysis. This is true whether the red blood cells are suspended in isotonic NaCl or in Locke's solution. The inorganic phosphates of the glycolyzing blood, however, invariably increase under the influence of arsenate, and in direct relation to the arsenate concentration, owing to the stimulation of the blood phosphatases. The effect seems to be quantitatively greater in isotonic NaCl than in Locke's solution.

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## DIET AND THE BLOOD LIPIDS

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There is in general a fair agreement between workers as regards the lipid levels of the blood, but there are occasional groups of "normal" values for the lipids, both of whole blood and plasma, which are out of line, and even in a single series occasional widely discrepant values may be found. Inadequacy of methods, while undoubtedly responsible for some of the differences, does not account for all, and even with the improvement of methods, discrepancies continue to appear. Cholesterol has in general been found to be more frequently and widely variable than other lipid constituents of blood, partly because more attention has been given to it, but mainly because there seems no question that it does vary greatly. Thus in a recent paper (1) the total fatty acid values for normal human subjects were practically the same as those given by me 15 years ago, determined by a method entirely different in principle, while the cholesterol values were much lower although the method was essentially the same. Another example of an unexplained difference in lipid level may be noted in the phospholipid content of the plasma of the dogs used about 4 years ago for work on diabetes in this laboratory with that of the dogs used in the present work. Both groups were normal healthy dogs but the former had an average phospholipid content of 400 mg. per cent; the latter averaged 70 mg. per cent. Also, as may be seen from Table I, the phospholipid value for the plasma of the dog reported more than doubled in a few months. An investigation of the factors which affect the lipid content of the blood was desirable.

It has been emphasized recently for other blood constituents (2) that the distribution of constituents between the corpuscles

and plasma both as regards values at equilibrium and as regards permeability must be taken into account in any blood analysis. As regards lipid constituents, Mayer and Schaeffer (3) have shown that the corpuscles behave as tissue cells, *i.e.* have a fairly constant composition, not only in the same species but in widely different species, while the content of the plasma is often widely different in different species, the plasma of herbivorous animals having in general a lower lipid content than that of carnivorous animals. This fact is important in itself and because it emphasizes the desirability of working with plasma instead of whole blood in the study of the lipids. On the other hand, there is evidence to show that there may be a temporary loading of the corpuscles with fat and phospholipid if there is a large inflow of fat into the blood, so that the effect of the corpuscles cannot be ignored.

Among the other factors influencing the blood lipids, food and especially food fat is obviously important and a number of studies have been made on this factor. Most of the investigations on the effect of food have had to do with single feedings but in some instances the effect of continuous feeding has been kept in mind. A single feeding of fat to carnivorous or omnivorous animals results in an increase in the blood of not only fat but also phospholipid and generally cholesterol. A single feeding of fat to herbivorous animals, *e.g.* the rabbit, may have no significant effect on the blood lipids, while continued feeding does produce increased plasma fat. The effect of fed fat on the blood fat is easily understood since the absorbed fat can be followed from the intestine by way of the thoracic duct into the circulation. The increase of phospholipid during fat absorption is explainable on the basis of its close chemical relationship to the fat. The increase of the chemically unrelated cholesterol has been referred to a participation in the transport or metabolism of the fat but it may have other and more general significance since it increases after non-fat food (4).

The visible effect of food fat on the blood lipids—alimentary lipemia—can be easily demonstrated in the dog, while it is difficult to produce in the rabbit. It can ordinarily be produced in man, but the amount of fat which can be assimilated at one dose is relatively much less than in the dog and the lipemia is generally less marked. Rony and Levy (5) have found that human

beings vary a great deal in the reaction of their blood lipids to a standard dose of fat, which they interpret to mean that some individuals have much greater tolerance for fat than others but the possibility of differences in rate of absorption in different individuals must be taken into account. Rony and Ching (6) found that alimentary lipemia could be prevented in dogs by feeding carbohydrate with the fat or by giving insulin. A similar effect of carbohydrate had been reported much earlier by Bang (7). Rony and Ching believe that the passage of sugar into the tissues facilitates the passage of fat.

The change in blood lipids as the result of fat feeding is to be expected. Changes as the result of feeding other foodstuffs such as carbohydrate or protein are more difficult to explain. Thus McClure and Huntsinger working with human subjects (4) have reported increases of cholesterol and total fatty acids after feeding any type of foodstuff, while blood sugar and amino acids could be increased only by feeding the corresponding carbohydrate or protein. The feeding of fat was found to lower the blood sugar.

Little attention has been paid to the effect of continuous feeding of substances on the blood lipids. Hunt (1) has examined the data in the literature with regard to the effect of diet on blood cholesterol and, while concluding that the weight of evidence was in favor of such an effect, was unable to demonstrate it in her own series. White and Hunt (8) found that overnutrition in children produced increased blood cholesterol and also that extreme variation from the normal body standard in weight was accompanied by high blood cholesterol.

The possibilities of diet or long continued feeding of a particular group of foodstuff as a factor influencing the level of various lipids in the blood was brought home to the writer by difficulties encountered in this laboratory by Glusker (9). On a fixed (low fat) diet he was able to obtain steady values for cholesterol and total fatty acids but his results for phospholipid were variable. Boyd (10) working with the same animals a little later was able to show that the reason for Glusker's difficulty was that the phospholipids in these animals were very low — in the neighborhood of 60 mg. per cent or one-fourth to one-fifth of the values ordinarily found in dogs. After verifying the low values of Boyd, the writer took over the four dogs having the lowest phospholipid

values, partly with the intention of finding out if possible the reason for the low values and in general of studying the effects of long continued feeding of various diets on the blood lipids. For comparison similar experiments were carried on with rabbits — typically herbivorous animals. Typical results of these experiments are given below. In brief, it may be said that the plasma lipids of rabbits could be made to pass through enormous variations in value depending on the fat content of the food, while the changes in the plasma lipids of the dog, although noteworthy and significant, were much less extensive.

#### EXPERIMENTAL

*Dogs*—Dogs 29-199, 29-209, 29-210, and 29-288, used by Glusker and Boyd, and in addition Dog 30-141 were the animals used. The first four had been on a diet of dog biscuit having a composition of crude fiber 2.14, fat 2.36, ash 8.61, protein 17.91, and carbohydrate 61.81 for several months — a low fat diet. In the present work they were first given for 16 days a diet of kitchen scraps of variable composition but containing considerable fat. Blood samples were taken on the 11th and 16th days. This diet caused a marked rise in the phospholipid values with lowering in cholesterol and fat (see Table I). The animals were then returned to the dog biscuit diet for a period of about a month, blood samples being taken about three times a week. At the end of that time one-third of the dog biscuit was replaced by an equidynamic amount of lard. This feeding was continued for 1 month, blood samples being taken as usual, after which the dog biscuit diet was resumed for 6 weeks, then the fat diet for about a month, and finally the dog biscuit diet for about 2 months. In brief, the animals were alternated between a low fat (fat = one-sixteenth of the caloric value of the diet) and a moderate fat diet (fat = one-third of the caloric value of the diet) over several months time. They remained in good condition, gaining slowly in weight throughout all the periods except the last one.

*Rabbits*—Five rabbits were alternated for varying periods between a fat-poor diet of hay, alfalfa, and cabbage, and a fat diet consisting of sunflower seeds of which the kernels contained about 40 per cent of fat. These animals also gained in weight slowly during the time.

The analytical methods used were those already described (11). Because of the low lipid levels in these animals 8 cc. samples of plasma for the dogs and 7 cc. samples for the rabbits were used for each 100 cc. of the alcohol-ether and the aliquots of the extracts were chosen so as to give the optimum amount of material for analysis. Samples were taken about 16 hours after the last meal. (The uneaten food was removed from the rabbits at 4 p.m. and the samples taken the next morning. The dogs generally ate up their food clean at once; what was not eaten at once was taken away.) Duplicate determinations were made on most samples, and were carried out some days apart so as to avoid the exact duplication of working conditions which will sometimes result in closely agreeing values both of which may be wrong. Direct measurements were made of total lipid, phospholipid, and cholesterol. The value for total fatty acids was obtained by subtracting the value for cholesterol from that of total lipid. Residual fatty acid (probably largely fat) was obtained by subtracting from the total fatty acid the values for the fatty acids in the phospholipid (two-thirds of the weight of phospholipid) and cholesterol ester ( $0.44 \times$  the weight of the plasma cholesterol<sup>1</sup>). Summaries of the results on one dog and one rabbit are given in Tables I and II below. Results on the other animals were of the same nature and so are not given.

*Periods 1 and 2*—Period 1 represents the condition of the blood lipids in the animals after they had been on the dog biscuit diet for several months (in use by Glusker and Boyd). The phospholipid value was abnormally low and the residual fatty acid value abnormally high. Cholesterol was low but not much below some of the values found later. What may be the significance of these abnormal phospholipid values is not known since it has not been possible to bring the animals back to this condition by long continued feeding with the dog biscuit diet. The mixed diet of kitchen scraps raised all the lipids but especially the phospholipid which reached values 3 times the low initial level.

<sup>1</sup> Six-tenths of the cholesterol in plasma is combined with fatty acid. Assuming that the fatty acid is oleic, the relation of cholesterol to fatty acid in the cholesterol ester molecule is 384:282. From these two values the fatty acid combined with cholesterol in the blood plasma is calculated to be 0.44 times the weight of the cholesterol present.

Returning the dogs to the biscuit diet brought the levels down again but not to the low beginning levels.

*Periods 3 to 7*—"It is common statistical practice to assign significance to a difference between means only when this difference exceeds one and one-half times the sum of the mean deviations

TABLE I  
*Effect of Diet on Blood Lipids of Dog 29-209*

Period No.	Time	Diet	Samples	Phospholipids	Cholesterol	Residual fatty acids	Total lipid
	days			mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	Beginning	Low fat	1	59	67	283	409
2	11	Mixed	1	184	86	334	604
	5	"	1	206	104	206	516
3	32	Low fat	12	High 175 Ave. $142 \pm 11$ Low 111	96 $68 \pm 6$ 50	200 $148 \pm 24$ 120	459 $365 \pm 53$ 330
4	25	Medium fat	9	High 202 Ave. $180 \pm 13$ Low 152	90 $80 \pm 6$ 71	268 $167 \pm 24$ 130	442 $402 \pm 22$ 363
5	49	Low fat	12	High 169 Ave. $157 \pm 7$ Low 145	106 $90 \pm 11$ 70	141 $116 \pm 16$ 94	397 $360 \pm 20$ 332
6	21	Medium fat	5	High 190 Ave. $181 \pm 6$ Low 171	122 $107 \pm 11$ 90	173 $138 \pm 19$ 110	455 $421 \pm 28$ 390
7	37	Low fat	14	High 155 Ave. $131 \pm 13$ Low 119	85 $72 \pm 6$ 62	118 $80 \pm 18$ 42	305 $275 \pm 20$ 247

The average deviation of the mean during the periods is given after the average value.

The average deviation of mean in per cent for the whole of the last five periods was phospholipids 6.6 per cent, cholesterol 9.0 per cent, residual fatty acids 16 per cent, total lipids 8 per cent.

of the respective means" (12). On this basis most of the differences between the means in the above groups would be without significance except in the case of the phospholipids. Cholesterol, residual fatty acids, and total lipids except between Periods 6 and 7 fall within this value. On the other hand, the differences in the case of phospholipid with the exception of Periods 4 and

5 are significant, and, furthermore, all the differences between the series repeat themselves at every shift from low fat to medium

TABLE II  
*Effect of Diet on Blood Lipids of Rabbit 1\**

Period No.	Time	Fat in diet	Phospho-	Cholesterol	Residual fatty acids	Total lipid
			lipids	mg. per cent	mg. per cent	mg. per cent
1	7	Low	75	26	183	284
2	7	High	100	52	304	456
3	7	Low	102	57	161	320
4	7	High	145	48	60	253
	14	"	133	46	131	283
	21	"	125	41	200	366
5	7	Low	35	25	90	150
	14	"	30	31	94	160
6	7	High	93	42	100	224
	14	"	98	50	134	274
	21	"	160	60	102	298
	28	"	143	50	99	270
7	7	Low	57	23	60	133
	14	"	10	15	100	128
	21	"	16	18	100	136
8	2	High	76	38	124	228
	9	"	128	54	190	357
	16	"	144	40	186	342
	23	"	177	61	170	380
	30	"	177	91	144	395
	37	"	190	94	119	386
	44	"	185	65	157	358
9	2	Low	72	43	146	259
	9	"	45	18	67	124
	16	"	67	31	82	173

\* This table is a continuous record.

fat diets (except cholesterol between Periods 4 and 5 in this dog. In the other dogs there were also irregularities in the cholesterol shift.) Because of this regular shift with the diet the differences

are believed to be significant. Especially is this the case in the phospholipid since the differences between the periods in the case of this constituent are mostly significant. There is little doubt therefore that the blood lipids in the dog do respond to changes in the level of fat in the diet but the changes are not extensive. They are most marked in the cases of phospholipid.

*Individual Variations*—Glusker and Boyd found that their dogs varied among themselves a great deal on the biscuit diet and not all the dogs showed the very low phospholipid values. The four dogs used in the present work were chosen because they did have low phospholipid values. Their behavior when the diets were shifted in the present experiment was similar, although in Dog 29-199 the changes in lipid values were less than in the other dogs. In the others the shifts in level were about the same in extent. Dog 30-141 was a young dog which had not been in the original group. The level of its blood lipids was much higher than that of the other dogs but the shifts in level on the different diets in the present series were similar to those of the others. It should be recorded that in this dog, when first put on the high fat diet after a period on low fat, all the lipid constituents of the plasma rose steadily throughout the period, the maximum increase for fat being greatest (70 per cent), the phospholipid next (60 per cent), and the cholesterol least (40 per cent). In other experiments the changes in values were much the same as in the other dogs.

The experiments with the rabbits furnish a more striking picture of the effect of fat in the diet on the blood lipids. A typical set of results is shown in Table II.

*Total Lipids*—The change in total lipid in the rabbits in response to the two diets was often very great. In the fat feeding Period 8, for example, they reached values 3 times those of the preceding low fat period. The promptness of the response, however, varied greatly. In Periods 1, 2, 3, the responses to change while notable were not as great as those found later — the mechanism of adaptation to the changes in diet was apparently not yet well developed. Beginning with Period 5 the changes in total lipid with change of diet became marked.

*Residual Fatty Acids*—This fraction is probably made up

largely of neutral fat. Since it is a value calculated from the other three, it is influenced by the errors of all of them and probably for this reason shows wide variations with, however, a tendency to adjust itself to a value of about 100 mg. per cent no matter what the diet.

*Cholesterol*—Especially in the later periods the cholesterol values vary widely with the diet, going up with the high fat diet and down almost to the disappearing point on the low fat diet. Its relation to the metabolism of fat in these animals seems definite.

*Phospholipids*—The phospholipid values vary greatly with the fat in the diet, going up with the high fat diet and down to very low values on the low fat.

The changes in the blood lipids with the diets are thus mainly in the phospholipid and cholesterol and seem to be directly referable to the amount of fat passing through the organism. It takes time for the adaptive mechanism to get into operation, a fact which is observable not only in the experiment as a whole but in the individual periods. This lag in individual periods can be clearly seen in the last two periods, in which 2 days on the new diet did not produce the maximum response. In these periods also can be seen evidence of overcompensation — a maximum response followed by an adjustment to an intermediate value.

*Cholesterol Esters*—Examination of the cholesterol-cholesterol ester balance was made in the combined residual extracts in both groups of animals. The results show that in the dogs the percentage of the total cholesterol present as ester is always higher on the high fat diet than on the low, while in the rabbits there is little difference. Thus, in the dogs the following values are found.

Dog No.	Low fat	High fat
	per cent as ester	per cent as ester
29-199	65	76
29-209	51	66
29-210	54	78
30-141	44	51

In the rabbits low fat and high fat are 48 and 44 per cent respectively.

## DISCUSSION

A considerable species difference in behavior toward fat in the diet is to be observed in these experiments. In both species, increasing the proportion of fat in the diet results in increased plasma phospholipid and cholesterol, but in the dogs the increase is inconsiderable, while in the rabbits it is great. The following factors should be considered in determining the significance of this difference in behavior.

(a) The dog, a carnivorous animal, is racially accustomed to a high content of fat in its food, is known to be able to digest and absorb large amounts of fat, and to burn it without waste. The rabbit cannot absorb fat rapidly, as shown by the fact that it is difficult to produce an alimentary lipemia.

(b) The phospholipid and cholesterol levels in dog plasma are characteristically much higher than those in the rabbit but in these experiments the rabbits, after being on a high fat diet for a week or more, develop plasma lipid values comparable with those of the dogs.

(c) In the rabbits, after the high fat diet is exchanged for a low fat diet, the plasma lipid values fall rapidly to very low levels. In the dogs the change from high fat to low fat produces relatively little change in the plasma lipid levels. The high lipid levels in the rabbit are thus connected directly with a high fat intake; in the dog connection is much less definite. The higher levels in the dog may probably be referred to the habitually higher fat diet of the species and it is possible that if rabbits were kept on a high fat diet long enough there would be the same continuously high level of blood lipids as in the dog.

Two possible functions of phospholipid and cholesterol may be mentioned in accounting for these changes in lipid levels with diet. (1) The two substances aid in the transport of fat. (2) They are fat metabolites — substances formed from the fats and necessary as stages in their utilization. Either of these functions would explain the phenomena observed in these experiments and also most of the findings in the literature. Whether they are agents in the transport or stages in the metabolism of the fats, it is to be expected that their level would be higher when much fat is passing through the blood than when there was little; thus it has been found that whenever there is a high content of fat in the

blood, as in alimentary lipemia or in the lipemias observed in diabetes and in the hemorrhagic anemia of rabbits, and whether the increased blood fat originates in the food or in the fat stores, there is found along with the high content of fat a high content of phospholipid and cholesterol. As stated by Klempner (13) years ago, a lipemia means a lipoidemia. It has also been found that a diet habitually high in fat results in a higher level of phospholipid and cholesterol in the plasma. This is the case with severe diabetics who must depend largely on fat for their energy. It was found to be true at least for cholesterol in the case of the two arctic explorers V. S. and K. A. when they lived experimentally on a high fat diet for a year (14). The actual fat of the blood is undoubtedly a metabolite — a substance in process of utilization either by combustion, chemical transformation, or storage. Whether phospholipid and cholesterol (at least cholesterol esters) are to be so regarded is not made clear by these experiments. Their increase during the actual absorption of fat might be related either to their function as metabolites or to that of aid in transport. The finding in the rabbit that removal of fat from the diet results in a prompt and great fall in both phospholipid and cholesterol might be explained either way. The fact that in the dog the fall is inconsiderable may perhaps be referred to different metabolic habits in the two animals. The dog bolts its food and thus floods its organism with the products of digestion. If its food is largely carbohydrate as in the biscuit diet, the rapid and large inflow of dextrose would tend to exceed the capacity for storage as glycogen and would result in a considerable formation of fat in order to avoid waste of carbohydrate. This fat would be transported and stored and since the animal is on a maintenance diet would later be removed from storage, transported, and burned, thus appearing in the blood twice in one day. The dog, although on a carbohydrate diet, may really be metabolizing a considerable proportion of its food as fat. Perhaps also the dog as a race has an inherited or developed preference for handling carbohydrate stuffs as fat, while the rabbit organism "prefers" to handle them as carbohydrate. In the rabbit and herbivorous animals generally, digestion and absorption are more continuous and relatively slow owing to the nature of the food and the greater length of intestine necessary to take

care of it. On a carbohydrate diet the inflow of dextrose would be slow, little faster than it could be disposed of by combustion but no faster than it could be taken care of as glycogen. Hence there is little or no food being metabolized as fat and no need for a high plasma content of phospholipid and cholesterol, whether they be regarded as aids in transport or as metabolites.

As noted above and as may be seen from Tables I and II the changes in blood cholesterol do not follow the changes in fat in the diet as closely as does the phospholipid. Cholesterol undoubtedly has other functions than that in fat metabolism.

#### SUMMARY

It has been found that the continuous feeding of diets, on the one hand high in fat and on the other hand low in fat, produces definite changes in the level of phospholipid and cholesterol in the blood plasma of dogs and rabbits. The levels of these two constituents on the high fat diet are always higher than on the low fat. The difference in levels is not great in the dog but in the rabbit it is very marked, especially after several periods of alternate high and low fat feeding. The significance of these changes and of the species difference is discussed and a possible difference in the way the two animals make use of carbohydrate in metabolism is indicated.

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## STARCH HYDROLYSIS AS AFFECTED BY LIGHT. II\*

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### *Diastasic Hydrolysis of Starch in Relation to Light*

Under the conditions described in a previous article (Navez and Rubenstein (8)) no significant difference was detected in the action of ordinary light and of plane-polarized light on the diastasic hydrolysis of solutions of soluble starch, as estimated by the time course of the formation of maltose. There was described a definite accelerating effect of light on the rate of hydrolysis, provided the purification of the amylase preparation took place completely in the dark or under very dim red light.

This distinction of "dark" and "light" reactions led us to consider the possibility that the light reaction might be sensitized by the addition of dyes known for their photodynamic action, thus giving data on the steps in the mechanism involved. It might also be considered that ordinary and plane-polarized lights might differ in their mode of action in the presence of the sensitizing dye, free or bound to some part of the reactant complex. It might happen, for instance, that a difference (if any exists) between the effects of the two types of light on the hydrolysis, too small to be detected by our procedure, could be increased to such a magnitude that it would be clearly evident.

Our procedure of exposure to radiation consisted in using two horizontal beams of light (one, of ordinary light; the other, of plane-polarized light) and reflecting them totally by means of two mirrors placed at 45° so as to have them impinge normally on the bottoms of two cells containing the reactant mixture. These cells, with a third identical one kept in total darkness, were placed in a thermostat provided with two plate glass windows in the bottom, through which the radiation passes.

\* This work was presented at The Thirteenth International Physiological Congress at Boston in 1929 (*Am. J. Physiol.*, 90, 460 (1929)).

In the experiments here reported a few minor improvements on our previously described technique were employed. A water thermostat kept the temperature ( $22.5^{\circ}$ ) constant to within  $\pm 0.02^{\circ}$ . We replaced the two silver mirrors by two thick plates of brass, heavily chromium-plated and brought to a high optical polish. This change obviates the very considerable work of obtaining highly reflecting silvered deposits, which are subject to rapid tarnishing. For the flat bottom Pyrex Erlenmeyer flasks used in the first part of the work, we substituted cells made from wide necked funnels of Pyrex glass sealed at the funnel end to thin discs of plate glass. The cement was tested as to possible reaction

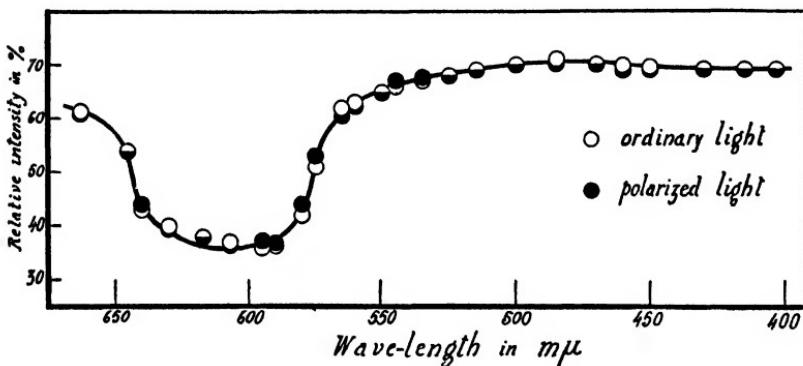


FIG. 1. The relative intensities of narrow, adjacent regions of the spectrum of the ordinary light and of the polarized light, expressed as percentage of the intensity of the same regions in the spectrum of a standard lamp.

with any of the substances in contact with it, but none was detected; care was taken to reduce the quantity of cement employed to the minimum, all excess of it inside the cell especially being avoided.

The same Nicol prisms were used as in the earlier experiments, and the same type of Mazda lamps. For both cases (ordinary light and polarized light) an intensity of illumination of 550 meter-candles was obtained. These figures refer to the effective intensity of light at the place where the hydrolysis was performed.<sup>1</sup> Fig. 1 (from spectrophotometric data) shows that the two beams

<sup>1</sup> In a few cases we used illuminations of the order of 3300 meter-candles with the same results as are described.

of light impinging on the reaction cells were as similar as possible as regards intensity in each spectral region. The rest of the technique was the same as previously described, all the diastase preparation being made up in darkness. For Benedict's method of sugar determination we substituted Willstätter and Schudel's iodometric titration, which gives a better end-point and is a little faster than Bertrand's method. The two last methods were very carefully compared for the titration of the hydrolyzed solutions and were found to be in very good agreement when carefully performed.

#### *Hydrolysis in Ordinary and in Polarized Light*

With the new technique, and in order to check the state of preservation of our crude diastase powder (kept constantly in the refrigerator), parallel runs were again made with radiation by each of the two types of light at an intensity of 550 meter-candles, a third aliquot portion being kept in the dark as a control.

The curves thus obtained for the time course of the hydrolysis are superimposable upon those obtained formerly. They reproduce the results found with the other apparatus. The same acceleration due to the addition of light to the system starch-diastase was again found, which is interesting when we consider that the intensity of illumination is here about twice as large as in the first case (267 meter-candles). This leads us to think of a saturation effect of light, saturation being attained with the intensity of about 267 meter-candles used previously. The other intensity used in the earlier work, *i.e.* 50.6 meter-candles, is, on the contrary, below the saturation point. Tests were performed with intensities of light below and above 270 meter-candles to check this saturation point. We found that a decrease of 10 per cent below 270 meter-candles in the intensity determined a decrease of about 5 per cent in the light effect. Therefore in the subsequent experiments the intensity of illumination was always kept above the saturation intensity.

The repetition of our previous experiments with the improved technique enabled us also to check the possibility that the plane of polarization could affect the course of the hydrolysis. The positions  $0^\circ$ ,  $45^\circ$ ,  $90^\circ$ , and  $135^\circ$  of the plane of polarization were tested with identical results. This lack of specific effect of any particular

direction of the plane of the polarization is in agreement with the random distribution of the particles that we must consider in the solution of soluble starch and diastase. For any position of the plane of polarization we ought to find the same number of particles placed in a critical position as regards this plane. As the suggestion could be made that a possible effective angle would be the angle of rotation of the solution in the reaction cell, or its complement, one may point out that the system starch-diastase has no constant angle of rotation of the plane of polarization, on account of the hydrolytic splitting. Furthermore, the light which is acting is far from being monochromatic, as it ranges from 420 to 700  $m\mu$ ; under these conditions no well defined value for the angle of polarization can be found. We must have then a distribution of effective rays in the light as well as a random distribution for the positions occupied by the starch-diastase particles. One has to remember also that the viscosity of the mixtures here used is practically that of water, so that we may not think of any special distribution of stresses in any particular direction, as in the case of films of colloidion on glass plates studied by Weigert (15). Furthermore, the "Weigert effect" is obtained with much lower light intensities than the ones here used.

It has been reported by Claus (3) that in the case of diastasic hydrolyses performed at constant temperature, with addition of dyes, a very marked change in the pH of the reacting mixture was found (extreme case, from pH 5.6 to 6.7). The pH shifted systematically during the course of the hydrolysis in a way dependent on the dye used. This is strange if one considers the quantity of buffer solution added to his hydrolytic mixture (5 cc. of buffer for 13 cc. of hydrolytic mixture). It can only be accounted for by the photolytic generation of a very large quantity of hydroxyl ions, whose origin must be traced to the dye and not to the hydrolysis *per se*. In our case electrometric determinations of pH were made with the H electrode and with the quinhydrone electrode, and no definite shift in pH could be detected. This is true also for the experiments in which dyes were used, as reported later. The fact that we used in our experiments the McIlvaine citric acid-phosphate buffer mixture is not the determining factor which prevented this reported shift in pH, as we also used the acetic acid-acetate mixture of the same pH (4.8) and could find no differences

in the results. We may perhaps attribute to our process of purification of the enzyme the removal of the product which apparently suffered photolysis in Claus' experiments. We feel that the reported shift of pH has nothing to do with the hydrolytic part of the reaction, but must be a secondary effect.

The photic reactivity of some constituent of the system starch-diastase led us to try to sensitize the reaction by the use of suitable internal colored screens added to the solutions. If a process of photosensitization takes place during the period of radiation, it is obvious that the addition of any substance able to accelerate the sensitization ought to act also as an accelerator for the hydrolysis. One could suppose, for instance, that light is acting to destroy a substance whose presence inhibits to a certain extent the progress of hydrolytic reaction. If that substance were destroyed at a faster rate after addition of a sensitizer, we might expect the time curves of hydrolysis to be steeper. The change in rate with different substances and under different conditions of radiation and hydrolysis might enable us to determine some steps in the chain of processes underlying the effect. Furthermore, it might be considered that in the presence of polarized light and ordinary light of the same intensity differential effects could perhaps be obtained upon addition of fluorescent dyes.

Substances like quinine sulfate must be discarded on account of their "toxic" effect on the hydrolysis. Certain other substances well known for their sensitizing properties were non-miscible with our liquids, or act in regions of the spectrum outside our usable range; we were thus brought to use dyestuffs showing their effect at such concentration that their addition would not "poison" the enzymatic process. Among these, fluorescein (as Na salt), eosin (B and Y),<sup>2</sup> erythrosin, phloxine, rhodamine, rose bengal, are the most useful on account of the different regions of the spectrum in which they showed a definite effect. Unfortunately rose bengal exerted a strong toxic action on the diastase. The absorption spectrum of each of these substances is known (Uhler and Wood (14)); in every case spectroscopic and spectrophotometric determinations were carefully made, however, under the actual experimental conditions.

<sup>2</sup> Our samples of eosin were purified by successive solution, precipitation, and crystallization. This technique increased the photodynamic effect.

For the first set of these experiments we used exclusively fluorescein, as the sodium salt, at the following concentrations:  $M/100,000$ ,  $M/250,000$ ,  $M/500,000$ ,  $M/1,000,000$ ,  $M/5,000,000$ ,  $M/10,000,000$  of the final mixture.

All the preparations were made from a 0.5 per cent stock solution of the dye, kept in the dark; the use of successive portions of this solution which was aging did not introduce in the course of the hydrolysis any different effect from the one obtained with solutions freshly made up.

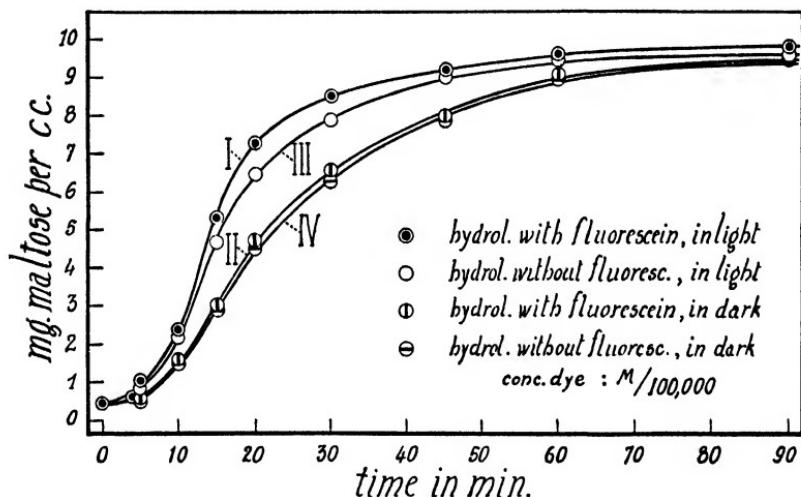


FIG. 2. The course of the hydrolysis of soluble starch by purified diastase under various conditions of illumination.

Three parallel runs were made: one under radiation by ordinary light, one with plane-polarized light, and a third aliquot portion (with dye) kept in the dark, all other conditions being similar for the three runs. The sugar determinations were made immediately after each run, special care being taken to stop the hydrolysis exactly at the end of the radiation period, the three portions of hydrolytic mixtures being poured simultaneously in 10 cc. of a 0.1 N iodine solution, preparatory step to the Willstätter-Schudel sugar titration.

The results are shown in Fig. 2, where Curve I represents the course of the reaction taking place in light and Curve II, the reaction

in the dark (each in presence of fluorescein); Curve III, the light reaction and Curve IV, the dark reaction, in absence of fluorescein.

As a notable feature of these curves we see that the addition of fluorescein to the hydrolytic mixture has in each case increased the rate of hydrolysis, without affecting the general shape of the curve. It has in fact almost doubled the maximum rate at which the process reaches a final equilibrium, the increase being more pronounced for the operation performed in light. A slightly higher percentage of hydrolysis (measured as maltose formed) is also to

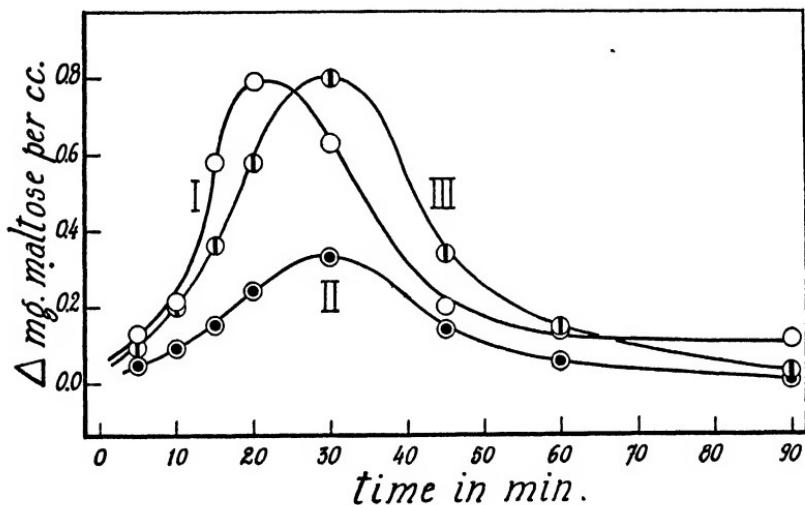


FIG. 3. Curves showing the differences between the hydrolysis curves with and without fluorescein (Na) in the presence or absence of light. Curve I, in the presence of light; Curve II, in absence of light; Curve III, the data of Curve II multiplied by 2.425 to bring the maximum of the curve to the same value as the maximum of Curve I, stressing in this way the difference between Curves I and II.

be noted, although this increase is small. No differences between hydrolysis performed in polarized light and in ordinary light could be detected, showing therefore that dye sensitization did not affect the diastase differentially in this respect. This effect of the fluorescein may perhaps be clearer if we consider the rate curves depicting the differences between the two processes (Fig. 3). Both are necessarily skew (from the shape of the hydrolysis curves),

but their maxima are reached at different moments, the one corresponding to the addition of fluorescein and light to the system being higher and sooner reached than the one corresponding to the same system with fluorescein in the dark.

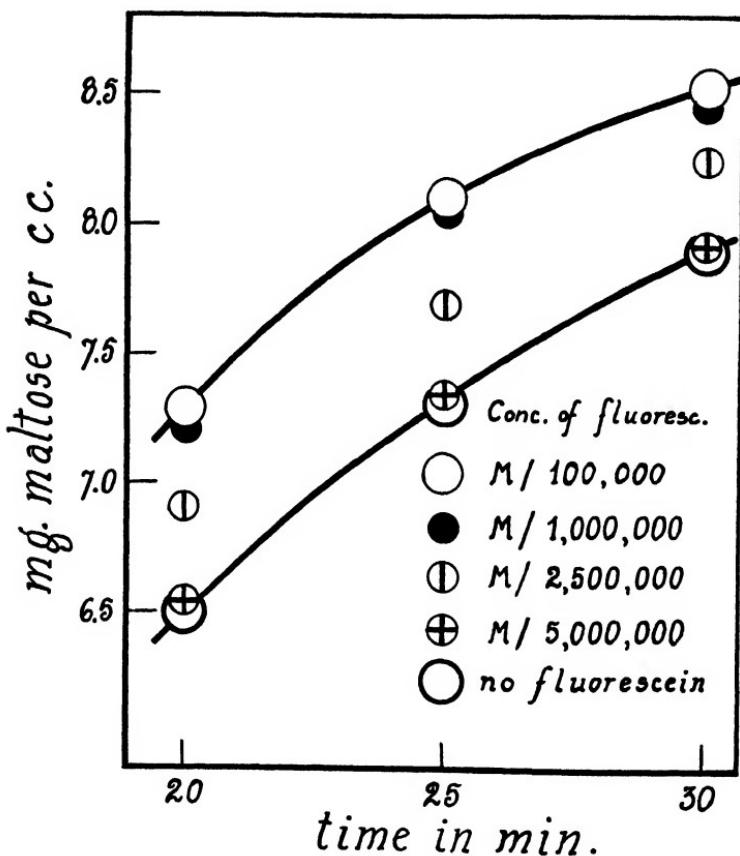


FIG. 4. The effect of various concentrations of fluorescein (Na) on the time curve of hydrolysis; only the region of maximal differences (20 to 30 minutes) is shown.

This points to a definite photosensitization by fluorescein when the concentration of the dye is sufficient (Fig. 4); the curve obtained for concentration of fluorescein  $M/5,000,000$  corresponds practically with the hydrolysis curve where no dyestuff has been

added. The curve for  $M/2,500,000$  fluorescein occupies a situation intermediate between the preceding and the one corresponding to  $M/1,000,000$ . The curve for  $M/100,000$  is almost superimposable upon the curve for  $M/1,000,000$ , and higher concentrations used do not further change the curves. Necessarily, some concentration could have been found which would depress the rate of hydrolysis; but we did not determine this limit. So we have here definite lower and upper limits of action: the phenomenon cannot be obtained for a concentration below a certain value ( $M/5,000,000$ ), nor is there any further effect when another (higher) concentration ( $M/1,000,000$ ) is reached. This effect is consistent with the idea of the formation of an internal color screen by the dye, effectively preventing light from reaching the uppermost layers of the reacting mixture. This may be comparable to the case described by Gros (5) in which the rate of oxidation of the fluorescein leucobase in the presence of tetrachlorotetrabromofluorescein passes very definitely through a maximum and further increase in the concentration of the photosensitizer has only a depressing action. Kistiakowsky (6) has suggested for this case that in the higher concentrations light is absorbed in the first thin layer and that the reaction is determined in part by the rate of diffusion of oxygen to the illuminated layer.

That dye and hydrolytic mixture must be mixed together (and probably unite in some unstable complex) can be shown by radiating the hydrolytic mixture (without dye) with light filtered through a trough containing the requisite concentration of dye. No activation is found under such experimental conditions. It can be shown also by radiating the solution of the dye alone, for different durations, adding it afterwards to the starch-diastase mixture, and allowing the hydrolysis to go on in the dark. No special activation of the diastase is exhibited. We may conclude for this reason that the dye alone is not transformed substantially to such a degree as to determine, on subsequent mixture with diastase, the activation of the latter.

On the other hand, we may radiate the enzyme without substrate, after addition of a dye, if we take care to perform the radiation at about  $0^\circ$ . Using such a radiated dye-enzyme complex, we may allow the hydrolysis to go on in the dark at  $22.5^\circ$  with the remarkable result of duplicating the curve of hydrolysis obtained

when we radiate the complete system, dye-hydrolytic mixture, in the usual manner at 22.5°. In other words, the enzyme can be activated separately by radiation, in the presence of the dye and in the absence of the starch substrate, to the extent reached normally in a complete system including dye and hydrolytic mixture.<sup>8</sup>

The foregoing facts point to the following conclusion: The dye reacts with the diastase, in presence of light, to form a complex which has a higher amylolytic activity than the enzyme radiated alone or than the dye-enzyme complex unirradiated. We have seen, on the other hand, that radiation of the enzyme alone increased its amylolytic power and that this activation could be accounted for by the destruction of an inhibitor.

In the case of the complete system, dye-hydrolytic mixture, we could thus conceive the total activation of the enzyme as being due to two partial activations: one corresponding to the enzyme plus light, the other being the one determined by the dye plus light. We may compare this case of activation with the one described by Noack (9), who showed that upon illumination, in the presence of a fluorescent dyestuff of the fluorescein series, the easily oxidizable substances present in aqueous extracts of *Vicia faba*, *Aloe socotrina*, etc., as well as in sections of living tissues of different organisms, were very rapidly oxidized (or dehydrogenated). The dye seemed to form an unstable peroxide, more active than  $H_2O_2$ , which can be inhibited in its action by the addition of reducing substances ( $Na_2SO_3$ , for instance).

We have seen that the dye-diastase complex was activated upon addition of light. This step could be interpreted as analogous to the formation of the unstable peroxide of Noack's case, probably due to the union of some constituent of the diastase with the dye, the necessary energy being supplied by the light. The existence of such a peroxide in our case is highly probable for the following reason: The addition of a reducing agent to the dye-hydrolytic

<sup>8</sup> This point is interesting in regard to some experiments by Blum (1, 2) on hemolysis by irradiated dyes. This author obtained an increase in the percentage of hemolysis by previously irradiated dyes. He concluded therefore that the dye was forming directly, *per se*, a peroxide which could bring about the hemolysis. In our case the peroxide or the activated molecules could only be formed after coupling the dye to the diastase. Blum has more recently given an extensive review of photodynamic effects (*Physiol. Rev.*, **12**, 1 (1932)).

mixture ought to prevent the activation due to the dye, by blocking the formation of the peroxide. This is what is found (Fig. 5), in fact, on addition of small portions of KI (making the final concentration of the reducing agent 0.001 to 0.01 M). In our experiments, dye-hydrolytic mixtures so treated showed in the light a curve of hydrolysis very close to the one obtained for hydrolysis without dye in the light. One could object that KI at such concentrations might interfere with the diastatic hydrolysis itself, but hydrolysis performed with and without KI in the absence of dye follows very nearly the same course.

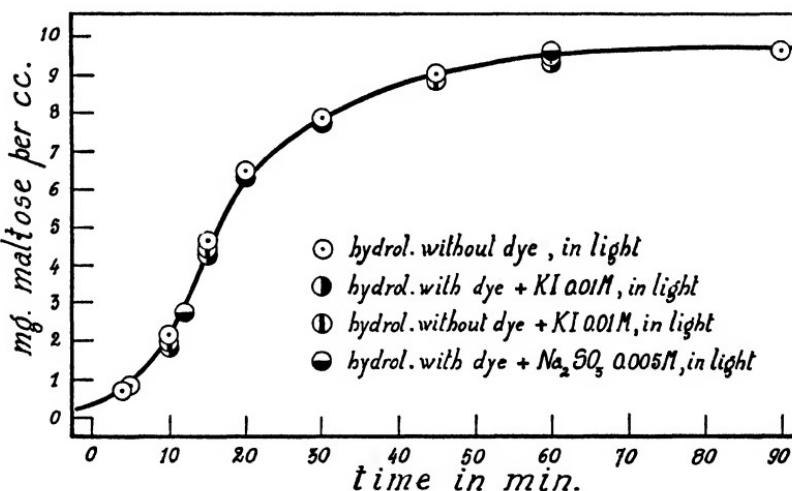


FIG. 5. The effect of additions of KI and  $\text{Na}_2\text{SO}_3$  to the hydrolytic mixture containing fluorescein, as depicted by the time curve. The three last series of points have been corrected for the reducing agent contained in the portions titrated.

The same effect can be obtained with  $\text{Na}_2\text{SO}_3$ . Very small traces of this salt inhibit completely the dye activation of the enzyme, without interfering at all with the course of the hydrolysis. Here again we might conceive the lack of activation as being due to a lack of formation of the dye peroxide. This is analogous to the instances described by Straub (12) and by Blum (1, 2). It presents over these two cases the advantage of being detectable after much shorter periods of exposure to light (of the order of 10 minutes instead of hours) and of involving a very widely occurring

biological reaction. But it differs widely from the one described by Blum in the fact that it is impossible to get any activation of the diastase by addition of radiated dye solution. In other words, the dye when radiated alone is not transformed to such a point as to determine activation of the diastase. We think therefore that this activation is a property of the diastase itself, brought about or simply magnified by its complex to a dye.

TABLE I

*Effect of Light of Different Spectral Range in Presence and Absence of Fluorescein*

The effect of light is expressed as mg. of maltose formed per cc. in 15 and 30 minutes and as percentage of the efficiency of white light, in the presence and absence of fluorescein.

Spectral range	Reaction in dark	Reaction in un-screened light	Reaction in screened light		Reaction in dark	Reaction in un-screened light	Reaction in screened light	
Without fluorescein; 15 min.								
<i>m<math>\mu</math></i>				per cent				per cent
440-590	2.87	4.71	4.20	89	6.25	7.89	7.27	92
510-590	2.85	4.70	4.35	92.5	6.23	7.86	7.38	94
540-590	2.85	4.72	4.46	94.5	6.28	7.90	7.50	95
628-707	2.88	4.70	3.97	84.5	6.25	7.93	6.81	86
With fluorescein m/100,000; 15 min.								
440-590	3.02	5.30	4.70	88.5	6.60	8.50	7.76	91
510-590	3.02	5.33	4.80	90	6.58	8.52	8.01	94
540-590	3.10	5.28	4.80	91	6.56	8.46	7.92	94
628-707	3.00	5.30	4.52	85	6.62	8.50	7.40	87
With fluorescein m/100,000; 30 min.								

It can be pointed out that the complicated dye-hydrolytic system is not equally sensitive all over the range of wave-lengths used. By the use of convenient screens one is able to cut out regions of the spectrum so as to follow their particular action. This we did, using among others the mixture of  $\text{CuCl}_2$ ,  $\text{CaCl}_2$ , described by Uhler and Wood ((14) p. 51), and the different dyes also used for sensitization.

From Table I it will be seen that certain narrow regions in the spectrum have efficiencies of activation nearly as great as the

white light used in the main sets of experiments. Nevertheless, the efficiency of the red end of the spectrum drops down very definitely. In each case we have determined the intensity of light transmitted in the zones considered and have used screens whose transmitted light was of the same order of intensity as the one of the corresponding region in the white light used; care was taken to keep the intensity of illumination at such a level that the drop due to the interposition of the color screens did not reduce the transmitted light to an intensity below the light saturation point already described.

In this way we have seen that the hydrolytic complex is sensitized by a dye in a region not necessarily corresponding with its maximum absorption. In fact, the region of maximum efficiency is situated at the limit of the absorption region, on the longer wavelength side;<sup>4</sup> perhaps this may be linked to the fact that the fluorescence of these same mixtures occurs in that region. It is to be noted also that these hydrolytic mixtures are slightly opalescent and show a well marked Tyndall effect.

All the foregoing statements have been made in relation to fluorescein; they can be extended to the other dyes used the only changes being in the absolute magnitude of the effect. The dyes can be arranged in decreasing order of effectiveness as follows: fluorescein, eosin Y-eosin B, erythrosin-phloxine, rhodamine.

#### DISCUSSION

In the diastase-starch mixture a substance partially inhibiting the hydrolysis is present. This inhibitor is equally sensitive to ordinary and to plane-polarized light, addition of light during the hydrolysis increasing the rate of liberation of reducing substances. This inhibitor is linked to the diastase complex, for irradiation of starch alone does not produce the same effect as irradiation of the total mixture. Irradiation of the enzyme alone, under definite

<sup>4</sup> This shift may be compared with the one reported by Eder (4) for the region of maximum photic sensitivity of the silver halide-gelatin complex of the photographic plate, after sensitization with the fluoresceins. The new maximum of sensitivity with fluoresceins lies further to the red by about 38 to 43  $m\mu$  than the maximum absorption region of the dye in aqueous solution, and by about 25  $m\mu$  when compared to the dyed emulsion. The magnitude of the shift in our experiments is of the same order (*cf.* (4) p. 35 and ff.).

conditions of temperature, activates the enzyme alone.<sup>5</sup> In the presence of dyes of the fluorescein series this activation is further increased. Here again the effect of the dye is on the diastase and not on the starch, for a starch solution irradiated after addition of the necessary amount of fluorescein does not show any activation when afterwards hydrolyzed by the enzyme in the dark. On the contrary, a diastase solution containing the necessary amount of dye will show activation when irradiated separately from the starch and subsequently allowed to hydrolyze in the dark. The efficiency of the white light used being taken as 100 per cent, we find that small regions in the center of the spectral range used have efficiencies of the order of 90 to 94 per cent; on the other hand, on the longer wave-length side of the spectrum the efficiency drops down to 84 per cent and even to 60 per cent (for the region 650 to  $707\text{ m}\mu$ ). The region of maximum efficiency corresponds, very nearly, to the limit of the regions between fluorescence maximum and maximum absorption.

The conclusions reached from the experiments performed with addition of dyes are important for the theory underlying this phenomenon. As it is known, two main theories of photodynamic action have been voiced: the first one, ascribed to von Tappeiner (13), is characterized by the fact that dye and substrate have to be radiated together in order to exhibit sensitization; in the other (see, for example, Ledoux-Lebard (7); Sacharoff and Sachs (11), etc.) it is considered that the dye radiated alone can show a specific photodynamic action in darkness when added to the substrate. Our case occupies an intermediate position between these two types. The diastase will not be activated by the radiated dye but the dye-diastase complex, after radiation, will show the photodynamic effect, in the dark, in its action on the substrate.

The majority of cases reported in the literature seem to fit the hypothesis of von Tappeiner. Blum's work (1, 2), on the contrary, tends to uphold the second hypothesis.

In this respect we may state that with the dyes that we had at our disposal we were unable to get indication of iodine liberation by

<sup>5</sup> This point is interesting when compared with Pincussen's results (10), with which it is in opposition. But in this author's descriptions many technical details are not given, making it therefore difficult to repeat the same conditions.

radiation of a fluorescein solution containing potassium iodide when the duration of radiation was less than 8 hours. The quantity of iodine liberated for such a length of radiation corresponds to 1 to 2 drops of a 0.02 N solution of sodium thiosulfate. As Blum has reported under the same conditions (*cf.* Blum (1) Table IV) quantities of iodine liberated corresponding to about 17 to 18 cc. of a 0.001 N solution of thiosulfate (= 0.34 to 0.36 cc., 0.02 N), we are inclined to think that in Blum's case either his dyes or his KI contained some substance which favored the photocatalytic process of iodine liberation. We might conceive that this substance would play here the same rôle that the diastase played in our experiments. If so, Blum's case would reduce itself to one similar to ours, enabling us, in fact, to extend von Tappeiner's hypothesis, expressing it then as follows: To show a photodynamic action the dye has to be radiated either in the presence of the substrate, or in the presence of another substance which, forming with the dye a loose complex, is able to act subsequently and with definite effect on the substrate in the dark. This loose complex shows peroxidic properties demonstrated by the possibility of inhibiting its effect by reducing agents (KI, Na<sub>2</sub>SO<sub>3</sub>). Both cases seem thus to be based in the long run on an oxidative process involving some constituent of the system considered.

We are indebted to the Milton Fund for Research, Harvard University, for aid in a portion of this work.

#### SUMMARY

A photosensitization effect can be produced in the hydrolysis of starch by diastase by means of dyes of the fluorescein series acting as internal screens. The effect is an increase in the rate of production of reducing sugars. Experimental evidence leads us to suggest the formation of a photosensitive loose complex between dye and diastase, which on addition of light acts as a peroxide capable of removing a diastasic inhibitor, possibly by oxidation. Indications point to the effectiveness of the spectral region situated at the limit between the absorption zone and the fluorescent region of the dyes used.

Ordinary and plane-polarized lights of the same intensity and spectral distribution have the same effect in this photosensitization.

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## MICRO COLORIMETRIC STUDIES

### III. ESTIMATION OF ORGANICALLY BOUND PHOSPHORUS. A SYSTEM OF ANALYSIS OF PHOSPHORUS COMPOUNDS IN BLOOD

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#### INTRODUCTION

In a previous paper (1), we pointed out the advantages of the method employed by Kuttner and Cohen (2) for the estimation of inorganic phosphate. In the present paper we are reporting the application of the method to the estimation of phosphorus in organic combination in general; and in biological fluids and tissues in particular. A system of analysis has been devised for the estimation of the inorganic and organic phosphorus compounds in blood; namely, inorganic phosphate, total acid-soluble phosphate phosphorus, ester, lipoid, and protein phosphorus.

One set of reagents is used for any or all of these estimations. The performance of the method and preparation of the necessary reagents are comparatively simple. The method is based upon the blue coloration produced upon the specific reduction by stannous chloride of phosphomolybdic acid, in the presence of a definite concentration of the reagents.

Kuttner and Cohen (2) used sodium molybdate in concentration of 0.75 per cent in the final mixture in preference to the ammonium salt. The latter may be substituted in the same concentration, but the color produced is then less intense. The concentration of reagents, as discussed in the previous paper (1), is convenient for a color range corresponding to 0.01 to 0.04 mg. of phosphorus in a final mixture of 10 cc. The color intensity within this range is satisfactory for the plunger and wedge type colorimeters.

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Smaller quantities of phosphorus, amounts between 0.0005 and 0.005 mg., can be estimated in Nessler tubes or a similar comparator type. In the method to be described we have found it practical to take samples, equivalent to 0.015 to 0.03 mg., of organically bound phosphorus. This comes within the aforementioned range for the plunger type colorimeter.

#### *Principle of Method*

The phosphorus-containing substance in solution, *e.g.* nucleic acid, blood, or tissue extract of suitable concentration, is digested in a miniature Kjeldahl tube with sulfuric acid aided by hydrogen peroxide. Water is added and the peroxide driven off by boiling. Finally a blue color is developed upon the addition of sodium molybdate and stannous chloride and estimated according to the method of Kuttner and Cohen (2) for phosphate.

#### *Description of Method*

The reagents required are prepared as in the previous paper of Kuttner and Lichtenstein (1), but the sulfuric acid and sodium molybdate are stored and used as separate solutions. The solutions necessary are:

*1. 10 n H<sub>2</sub>SO<sub>4</sub> or the Following Which Is Nearly 10 n and Will Give Satisfactory Results*—Pour 282 cc. of concentrated H<sub>2</sub>SO<sub>4</sub> (95 per cent, sp. gr. 1.84) into about 600 cc. of distilled water, cool, transfer to a liter flask, and make up to volume with distilled water.

*2. 7.5 Per Cent Solution of Sodium Molybdate*—Dissolve 7.5 gm. of sodium molybdate in water in a 100 cc. graduated flask and make up to volume with distilled water. A few drops of toluene may be added to prevent the growth of molds. Ammonium molybdate may be substituted for the sodium salt, if the diminished color production suffices. The purity of these salts has been discussed by us in a previous paper (1).

By storing and using the sulfuric acid and the sodium molybdate as separate reagents, it is not necessary to have at hand a number of mixed reagents containing the sodium molybdate and varying concentrations of sulfuric acid as is done by Fiske and Subbarow (3) and others.

*3. Stannous Chloride Stock Solution*—Dissolve 10 gm. of stan-

nous chloride in 25 cc. of concentrated hydrochloric acid. Smaller quantities may be prepared if desired. Store in a brown glass-stoppered bottle and keep away from heat. A surface layer of toluene may be used to prevent oxidation. We prefer to prepare the stock solution every 4 to 6 weeks, although it may keep longer. For immediate use only, dilute 1 part of stock solution in 200 parts of distilled water. Any unused portions should be discarded.

4. *Standard Phosphate Stock Solution*—Dissolve 0.4394 gm. of dried monopotassium phosphate in 1 liter of distilled water, and add a few drops of chloroform to prevent mold formation. 1 cc. = 0.1 mg. of phosphorus. Make two standard phosphate solutions by diluting 3 cc. and 6 cc. in 100 cc. graduated flasks and fill to the mark with water. The solutions contain 0.003 and 0.006 mg. of phosphorus per cc.

#### *Estimation of Organically Bound Phosphorus*

.*Procedure*—The substance to be examined should be in solution having a phosphorus equivalent of 1 to 4 mg. per 100 cc. Weaker solutions, alcohol-ether extracts for example, can be concentrated to 2 to 3 cc. in a beaker placed on a water bath or hot plate. The concentrate is transferred quantitatively to a 10 cc. glass-stoppered graduate. The beaker is rinsed several times with small portions of the solvent used and the rinsings are also added. The solution finally is made up to a convenient volume.

An aliquot portion which should contain about 0.01 to 0.04 mg. of phosphorus is transferred to a miniature Kjeldahl tube. The latter is non-lipped, of Pyrex glass, dimensions 16 × 180 mm., the lower end blown into a bulb of 5 cc. capacity. The tube is graduated to 20 cc. at 5 cc. intervals. 1 cc. of 10 N sulfuric acid is added, with a small chip of unglazed porcelain from a broken plate to prevent bumping. The mixture is digested over a micro burner. We use a Pregl apparatus<sup>1</sup> arranged for six micro-Kjeldahl determinations. The micro-Kjeldahl flasks for this apparatus have a capacity of 25 cc. but the special Pyrex tubes of 5 cc. bulb capacity also fit into the openings of the asbestos stand of the apparatus.

<sup>1</sup> This is obtainable at supply houses. (For description see Pregl, F., Die quantitative organische Mikroanalyse, Berlin, 2nd edition, pp. 113-122 (1923).)

The tips of these burners are metal but we find clay tips to be superior as they give a long pointed blue flame which is easily regulated.

After about 2 to 3 minutes, the material begins to char and soon dense white fumes appear. At this point the flame is reduced to a minimum. Several drops of hydrogen peroxide<sup>2</sup> are added until the mixture clears on continued gentle heating. This occurs in a few seconds. The heating is continued for a longer time to allow most of the peroxide to be driven off. A few tenths of a cc. of water are now added along the sides of the tube in a fine stream by means of a pipette drawn to a fine bore at the lower end. A rubber bulb may be attached if desired. The water is boiled to drive off the remainder of the peroxide. Care should be exercised not to allow the mixture to concentrate sufficiently to become yellow again. This process of adding more water and boiling is repeated about three more times. Traces of hydrogen peroxide interfere by an adventitious yellow, producing a green instead of a pure blue coloration, necessitating rejection. For this reason a minimum amount of peroxide should be used and great care exercised to drive it off completely.

After the tube has been removed from the flame and cooled to room temperature, distilled water is added to the 5 cc. mark. 3 cc. more are added, followed by 1 cc. of molybdate reagent. Finally, the dilute stannous chloride reagent is added. The tube is stoppered and inverted to mix the contents before the addition of each reagent. This procedure must be strictly adhered to.

We prepare the standards and unknowns up to the addition of the molybdate reagent and then, whenever convenient, add the stannous chloride simultaneously to both standards and unknowns. The colorimetric comparison can be made at once or within the following 2 hours in the usual manner according to the type of instrument used. Two standards are usually prepared containing 0.015 and 0.03 mg. of phosphorus respectively. If the unknown, however, contains 0.02 to 0.025 mg. of phosphorus, compare with a standard containing 0.02 mg. of phosphorus.

Regarding the range of proportionality we have found that when the phosphorus content of the unknown differs from that of the

<sup>2</sup> When 30 per cent hydrogen peroxide is used, 1 to 2 drops usually suffice.

standard by one-third of the latter or less, the intensity of the blue color can be considered directly proportional to the quantity of the phosphorus present in the unknown. The deviation from the theoretical values are within the error of the colorimetric method itself, *i.e.* 3 per cent. This is shown in Table I, which gives the range of direct proportionality for each of the standards suggested; namely, 0.015, 0.02, and 0.03 mg. of phosphorus. When the phosphorus content of the unknown differs from that of the standard by more than one-third, corresponding deviations from the "Beer Law" are noted for which corrections are indicated.<sup>3</sup> While this permits the use of a single phosphorus standard, we prefer, for accurate results, to use standards which approach the concentration of the unknowns as closely as possible even though it necessitates the preparation of additional standards.

TABLE I  
*Proportionality of Color Intensity*

• Phosphorus standard mg.	Range of unknown P mg.	Deviation from theoretical values per cent
0.015	0.01–0.02	<3
0.02	0.015–0.025	<3
0.03	0.025–0.04	<3

#### *Estimation of Lipoid Phosphorus*

*In Blood*—Treat 1 volume of whole blood, plasma, or serum as the case may be, with 50 volumes of Bloor's mixture (ether 1 part, alcohol 3 parts) in a graduated flask according to Bloor's method (5) for extraction of lipoid. Filter. A portion of the filtrate may be set aside if desired for the estimation of cholesterol and cholesterol esters. Concentrate 40 cc. of the filtrate to a volume of 4 cc. Transfer 1 cc. of the concentrate equivalent to 0.2 cc. of blood to the special micro-Kjeldahl tube and proceed with digestion, color production, and comparison as described in the procedure for the estimation of organically bound phosphorus.

<sup>3</sup> This was pointed out by Bodansky, Hallman, and Bonoff (4), who employed a single standard, in their paper which was published since this manuscript was written.

*In Tissues*—Tissues, both moist and dry, can be extracted with a variety of solvents. The extraction methods used by Sobotka, Epstein, and Lichtenstein (6), who applied this method of estimating lipoid phosphorus to the tissues in a case of Niemann-Pick's disease, proved satisfactory. They used acetone for the initial extraction of fresh tissue, followed by alcohol and ether. Enzyme action and oxidation processes are in this way inhibited. For the fractionation of the lipoids, the details of their procedure may be followed. The extraction of dried tissue or those fixed in formaldehyde is objectionable because of the danger of decomposition and autolysis in the former, and of hydrolysis in the latter case.

Each fraction is made up to a definite volume and a preliminary determination is made of the relative concentration of the lipoid phosphorus. An aliquot portion giving optimum concentration for color production is then used for the digestion and estimation. Care must be exercised in the digestion, as these mixtures occasionally cause frothing. This necessitates a slowing up of the process of digestion which we prefer to the use of an antifoam mixture. If much fatty acid is present during the digestion process, more than the usual amount of hydrogen peroxide may be necessary. A sample may be encountered having an unusually high fat or nitrogen content. It is then advisable to double the amount of 10 N sulfuric acid, using 2 cc. for the digestion; not forgetting to double the usual amount of sodium molybdate, in this sample as well as in the standards when making up the final volume of 10 cc.

*System of Analysis for Inorganic and Organically Combined Phosphorus in Blood*

*Estimation of Total Phosphorus*—Lake 2 cc. of oxalated blood with distilled water and make up to a volume of 10 cc. Take 0.3 cc. of the solution equivalent to 0.06 cc. of whole blood, digest with 1 cc. of 10 N sulfuric acid, and continue as previously described in the procedure for the estimation of organically bound phosphorus. Compare with a standard containing 0.03 mg. of phosphorus.

The remaining laked blood may be used for the estimation of total acid-soluble and inorganic phosphorus as follows:

*Estimation of Phosphorus as Total Acid-Soluble Phosphate*—To the remaining 9.7 cc. of laked blood add an equal volume of 7

per cent trichloroacetic acid. After complete precipitation of the proteins, filter or centrifuge. Digest 1 cc. of the filtrate, equivalent to 0.1 cc. of whole blood, with 1 cc. of 10 N sulfuric acid, proceeding in the manner described for the estimation of organically bound phosphorus.

The remainder of the filtrate is reserved for the estimation of inorganic phosphate.

*Estimation of Inorganic Phosphate*—Take 5 cc. of the trichloroacetic acid filtrate, equivalent to 0.5 cc. of blood, which was reserved from the previous determination, and transfer it to a test-tube graduated at 5 and 10 cc. Proceed by the addition of the sulfuric acid, the sodium molybdate, and dilute stannous chloride reagents and compare colorimetrically in the usual manner.

*Estimation of Phosphorus Combined as Ester*—The phosphorus combined as ester is ascertained by subtracting the inorganic phosphorus from the total acid-soluble phosphorus.

• *Estimation of Inorganic Phosphate in Serum or Plasma*

Transfer 1 cc. of blood serum or plasma to a test-tube graduated at 5 and 10 cc. Add 7 per cent trichloroacetic acid to the 10 cc. mark to precipitate the protein. Shake well, and, after 5 minutes, centrifuge. Transfer 5 cc. of the perfectly clear protein-free supernatant fluid, equivalent to 0.5 cc. of serum or plasma, to a similarly graduated tube.

Proceed by the addition of 1 cc. of 10 N sulfuric acid, 2 cc. of distilled water (mix contents), and 1 cc. of 7.5 per cent sodium molybdate solution. Close with a rubber stopper and invert. Then add 1 cc. of the diluted stannous chloride reagent to each tube. Immediately invert once or twice. Compare with the standard solution, prepared simultaneously, in the usual manner.

It is of importance to note that citrates when added to blood as an anticoagulant, seriously interfere with color production. This was pointed out by Kuttner and Cohen (2). However, we have found that oxalates do not interfere, even in considerably larger amount than that used as an anticoagulant. The presence of 10 times that amount of oxalate has no demonstrable effect upon the maximal color production, although there is a retardation of maximal color when 5 times the amount or more is present.

## DISCUSSION

The application of the method of Kuttner and Cohen (2) for the estimation of inorganic phosphate, to the estimation of phosphorus organically bound is practical and accurate when the procedure as described above is followed. The digestion as outlined in this method, does not entail sufficient loss of sulfuric acid as fumes to appreciably affect the accuracy of the determination. This is shown in Table II, which gives the colorimeter readings of a series of tubes each containing 0.02 mg. of phosphorus in a total volume of 10 cc., which were digested with sulfuric acid for the same length of time necessary for the organically bound phosphorus. This experiment was repeated several times with similar results. It is seen that the average deviation from the mean is less than  $\pm 1$  per cent.

TABLE II

*Colorimetric Readings of a Series of Digestion Tubes Each Containing 0.02 Mg. of Phosphate Phosphorus in a Total Volume of 10 Cc.*

Colorimeter readings mm.	Deviation from average per cent
15.1	+0.7
14.8	-1.3
14.9	-0.7
15.1	+0.7
15.0	0.0
14.9	-0.7

There is not sufficient silica dissolved during digestion to have any demonstrable effect upon maximal color production.

We chose as substance a synthetic stearic acid lecithin, (prepared by George Schicht, A. G. Aussig, Czechoslovakia) in alcohol-ether suspension, 1 cc. of which was equivalent to 0.02 mg. of phosphorus. Gravimetric determinations as magnesium pyrophosphate, with 100 mg. samples, show this substance to contain 95.0 per cent of lecithin. Our preliminary tests used in developing this method gave checks and concordant results with the gravimetric method. Six separate determinations indicated a lecithin content in the substance of 92.9 per cent average. This comes within 2.1 per cent of the gravimetric determination.

It was found that the phosphoric acid splits off with comparative

ease upon digestion. Lecithin equivalent to 0.03 mg. of phosphorus was digested by sulfuric acid alone without the addition of hydrogen peroxide in about 3 minutes. This was repeated with six more tubes, all of which checked within 3 per cent. Less sulfuric acid and a much shorter length of time is required than in the case of nitrogen determinations.

TABLE III  
*Estimation of Lipoid Phosphorus in Blood Plasma*

Diagnosis	No. of determinations	Mean values	Average deviation	Deviation from average
		mg. per 100 cc.		per cent
Renal tuberculosis.....	2	5.6	0.15	2.7
Capillary toxicosis.....	5	7.0	0.15	2.1
Acute nephritis.....	3	7.4	0.18	2.4
"    ".....	3	7.5	0.10	1.3
Chronic ".....	3	7.6	0.10	1.3
Acute ".....	3	7.6	0.03	0.4
Carcinoma of pancreas.....	2	7.8	0.09	1.2
Diabetes.....	3	8.1	0.07	0.9
Chronic nephritis.....	4	8.4	0.10	1.2
Pyonephrosis.....	3	8.4	0.10	1.2
Hemolytic icterus.....	2	9.0	0.10	1.1
Chronic nephritis.....	4	8.9	0.18	2.0
"    ".....	4	10.1	0.40	3.9
Uremia.....	3	10.3	0.10	1.0
Amyloid disease.....	3	10.4	0.10	1.0
Salvarsan hepatitis.....	4	11.5	0.20	1.7
Amyloid disease.....	2	11.8	0.30	2.5
Chronic nephritis.....	5	12.0	0.20	1.7
Diabetes.....	3	12.8	0.03	0.2
Nephrosis.....	3	12.9	0.25	2.0

Inorganic phosphate added directly to the lecithin digestion mixture gave values within 2 to 3 per cent of the theoretical phosphorus content. Likewise, varying amounts of lecithin added to alcohol-ether extracts of blood gave values within 2 to 3 per cent of the theoretical phosphorus content.

Inorganic phosphate added to blood is not recovered in the alcohol-ether extract. This is in accord with the original observation of Bloor (5).

To test further the reliability of the method, estimations of lipoid phosphorus in blood plasma were made in twenty cases of hospital patients, as shown in Table III. 64 separate determinations gave an average error of  $\pm 1.6$  per cent.

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## THE INORGANIC COMPOSITION OF THE PAROTID SALIVA OF THE DOG AND ITS RELATION TO THE COMPOSITION OF THE SERUM\*

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### INTRODUCTION

In studies on secretion much use has been made of saliva and the salivary glands, because of the ease with which this fluid can be obtained and the ready accessibility of the glands. Since the time of Ludwig, it has been known that the composition of saliva varies greatly with the type of stimulus. Clark and Shell (1) in an elaborate study found no correlation between diet and the composition of human saliva. However, Clark and Levine (2) reported that an increase in the phosphate concentration of the blood, following the ingestion of soluble phosphates, produced a large increase in the phosphate concentration of the saliva. But ingestion of sodium chloride resulted in only a small increase in the chloride concentration of the serum and no significant changes in the chloride concentration of the saliva. Baxter (3, 4) reported variations of ash, organic matter, and chloride which occurred during the progress of secretion. Werther (5), studying the submaxillary gland, showed that chloride and water-soluble salts (which were probably mainly sodium chloride and bicarbonate) were secreted in higher concentration when the rate of secretion was considerably increased. Gregersen and Ingalls (6) have recently demonstrated that sodium but not potassium varies in this way. The submaxillary saliva of the dogs studied was secreted after *chorda tympani* and pilocarpine stimulation.

\* This paper has been presented by Edwin J. de Beer to the Graduate School of the University of Pennsylvania as a thesis in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Wide variations occur in the composition of saliva obtained from different species. The bulk of the inorganic portion of human saliva consists of potassium and phosphate. Chloride, sodium, calcium, and carbon dioxide are present in fair amounts. The inorganic constituents of dog parotid saliva, as will be seen from our work, consist largely of sodium, bicarbonate, and chloride with small amounts of potassium and calcium. Furthermore, it has been found, not only that the salivary glands of different species of animals may produce saliva of widely different composition as regards inorganic constituents but also that the saliva produced by one individual may show considerable fluctuations.

In order to study the variations in composition of saliva in detail and to determine the effect of varying the composition of the blood circulating through the glands, the following experiments were carried out.

#### *Methods*

Normal dogs were anesthetized with amytal injected intravenously or intraperitoneally, and both of Stenson's ducts were cannulated. The cannulas were made by cutting off the tips of hypodermic needles of suitable bore, and polishing the ends. By means of adapters and capillary glass tubing, which was clamped firmly to the operating table, the cannulas were connected to graduated, mercury-filled tonometers. The animal was placed on its back and its dorsal jaw so fastened that the mucus and saliva coming from other glands would not drain down the throat. It was found that a free flow of saliva could best be obtained by distending the oral cavity and holding the cheeks stretched away from the gums during the experiment. Pilocarpine, dissolved in physiological saline solution, was administered in doses of 0.3 mg. per kilo of body weight, either intravenously by way of the external jugular vein or intraperitoneally. The intraperitoneal injection gave a slower but more uniform flow of saliva.

Four or more samples of saliva, usually about 15 cc. each, were collected from both right and left parotid glands. Usually one injection of pilocarpine was sufficient to obtain enough saliva for one sample. After two or more control samples of saliva had been collected, the salt solutions were injected intravenously, into the femoral vein, unless otherwise stated. Blood was collected

under oil from one of the external jugular veins at the beginning of the experiment, immediately after the injection, and at the end of the experiment.

Total carbon dioxide was determined on both saliva and serum immediately at the conclusion of the experiment by the method of Van Slyke and Stadie (7), with a calibrated micro volumetric apparatus. Since the amount of  $H_2CO_3$  in the saliva never amounted to more than 1 or 2 milli-equivalents, as can be calculated from the pH and total  $CO_2$  measured, and the quantity of  $Na_2CO_3$  was negligible, the total  $CO_2$  measured came largely from bicarbonate and has been calculated as such. The pH determinations were made immediately after collecting the sample. Since only relatively wide variations appear to be significant, determinations were made colorimetrically at 38°. Phenol red was used as the indicator, and standard buffers were prepared in tenths of a pH unit, according to Clark (8). Chloride was determined by Wilson and Ball's (9) modification of Van Slyke's method. Serum calcium was determined according to Clark and Collip (10).

Total solids were determined by drying overnight at 100°. All other determinations were made on ashed material. The saliva was ashed easily; large platinum evaporating dishes were used, and the procedure suggested by Stolte (11) was followed. The ash was dissolved by adding 1 cc. of 1.0 N hydrochloric acid, and made up to 10 cc. in small calibrated flasks. Aliquots were used for the following determinations. Sodium was determined by the method of Barber and Kolthoff (12) and potassium by the titration method of Shohl and Bennett (13). Instead of using a "glass bead filter" as described by the latter authors, it was found to be more convenient to use a filter prepared as follows: A small perforated platinum disk was supported on a shoulder ground in the apex of a 1 inch glass funnel, suction was applied, and an asbestos mat built up in the usual way. Calcium was precipitated with ammonium oxalate. The solution was made neutral to methyl red by means of dilute ammonium hydroxide, and after standing 2 hours the precipitate was filtered on the micro filters described above, washed with 3 cc. of 2 per cent ammonium hydroxide, then dissolved in 1.0 N sulfuric acid and titrated hot, with standard potassium permanganate.

The averages of duplicate determinations are given in Tables I to

V. In most experiments saliva was collected from both right and left parotid glands. Samples obtained at the same time from the two sides have similar composition unless the rates are very different. On account of this great similarity data are given for saliva obtained from one side only. Likewise, although two or more experiments dealing with each type of injection were made, only one typical example of each is reported in order to save space.

### *Results*

During the course of an experiment, although a great deal of fluid was lost from the animal, blood of fairly constant composition was circulating through the glands (see Table I). In typical experiments, dogs of from 15 to 20 kilos of body weight excreted from 75 to 150 cc. of parotid saliva and from 200 to 400 cc. of saliva from the other glands as well as from 100 to 300 cc. of urine. Defecation usually occurred. Meanwhile, the serum-cell ratio, as estimated by centrifuging, showed a progressive change from the usual 1:1 ratio, to values as low as 1:3 or 1:4. In view of the great loss of fluid from the animal, the relative constancy of composition of the serum is remarkable.

The data obtained in a typical control experiment are presented in Table I. Additional control data are furnished by each of the other experiments in which several samples of saliva were collected as controls before the injections of the salt solutions. The data so obtained serve not only as a basis for the interpretation of the results of the injections but also show the variations which may occur from individual to individual. The control data demonstrate that while saliva obtained from different dogs may vary in the concentration levels of inorganic constituents, the quantitative composition of all samples but the initial, from any particular animal, remains moderately uniform.

An inspection of the data from all experiments reveals that the sum of the measured inorganic anions, expressed as milli-equivalents per liter, is greater than that of the inorganic cations. This preponderance of acid is especially marked in the first sample. Since the pH ranged from 7.4 to 7.9, some base other than those studied must have been present. It is probably not mucin as this protein shows acidic properties and presumably binds base in the saliva.

## Dog 16, male, weight 22.8 kilos.

Sample No. ....	Blood serum			Saliva		
	1	2	3	1	2	3
Time of collection	10.15 a.m.	11.50 a.m.	1.40 p.m.	10.10- 10.25 a.m.	10.28- 11.15 a.m.	11.17- 11.57 a.m.
Volume, cc. ....	35*	40*	35*	R.	L.	R.
Rate, cc. per kg. per hr. ....				12.0	12.5	20.0
Solids, per cent....				13.5	15.0	12.0
Ash " "				2.1	0.7	1.3
pH.....				2.5	0.9	0.8
HCO <sub>3</sub> , m.-eq. per l.	26.4	23.8	21.8	R.	1.523	1.130
Cl " " "	110.3	107.5	106.6	L.	1.387	1.129
Na " " "	130.6	135.6	126.3	R.	0.911	0.851
K " " "	4.2	3.6	4.5	L.	0.883	0.859
Ca " " "	5.2	5.1	5.3	R.	0.841	0.853
				R.	7.7	7.7
				L.	7.7	7.6
				R.	57.7	54.3
				L.	62.6	56.3
				R.	103.7	100.1
				L.	98.8	99.9
				R.	130.2	123.4
				L.	127.6	116.7
				R.	12.3	11.1
				L.	10.5	8.6
				R.	9.4	8.4
				L.	10.3	7.8
						7.6

Pilocarpine was given intravenously at 10.03, 10.30, and 11.07 a.m.

\* Amount of whole blood.

In general, the initial sample of saliva was richer in total solids than subsequent samples. Baxter (3, 4) reports similar findings. This variation was due largely, though not entirely, to organic material, as is shown by the amount of ash. The difference, however, between total solids and ash does not give a true measure of the amount of organic material present, for during the ashing process there is a loss of inorganic material due to volatilization of ammonium salts if present and of part of the CO<sub>2</sub> of the bicarbonate. On the other hand, there may be small gains from the sulfur and phosphorus of the proteins.

The chloride concentration of the saliva, which was always lower than that of the serum, varied in the initial samples from different animals within wide limits; *i.e.*, from about 30 to 105 milli-equivalents per liter. There is a tendency toward a progressive drop in the chloride content of successive samples during an experiment. (See Table I.) This phenomenon at times reached much greater proportions than those shown by the control experiment. A slight decrease in the chloride concentration of the serum usually occurred simultaneously.

The total carbon dioxide concentration of the saliva is approximately double that of the blood serum. This constituent exhibited less variation among individuals than the chloride, and remained at a fairly constant level throughout the major part of the experiment. Occasionally toward the end of an experiment, an inexplicable rise in total carbon dioxide content of the saliva occurred, sometimes reaching values double those of the previous level. The total carbon dioxide concentration of serum usually showed a decrease during the course of the experiment.

The sodium concentration of the saliva was always (with the exception of one experiment (Table I)) lower than that of serum. It ranged from 48 to 133 milli-equivalents per liter. The fluctuations during an experiment were considerable, due partly, no doubt, to variations in rate of flow, as observed by Gregersen and Ingalls (6), in submaxillary saliva. An effort was made to keep the rate of flow uniform in our experiments, but with only occasional injections of pilocarpine this was obviously impossible. The figures quoted in Tables I to V represent average rates which in many instances were far from uniform for the period. However, in continuous experiments such as ours the rate of flow was probably

only one factor which influenced the concentration of the various constituents. For example, Table IV (Periods 1 to 4) shows variations in rates of flow and concentrations of constituents following one injection of pilocarpine. The rate of flow during the collection of the first sample was higher than during subsequent collections and the concentration of sodium as well as of all the other constituents was higher. In Periods 2, 3, and 4, when the rate of flow was not diminishing and was probably reasonably constant, the concentrations of sodium fell continuously. The same was true for the chloride. It is also worthy of note that the maximum concentrations of sodium observed in this study (Table I) were not associated with rates of flow above those in other experiments.

As a rule potassium concentrations were high for the first sample, lower for the second, and then tended toward a plateau level of 10 to 12 milli-equivalents per liter. These values are 2 to 4 times as high as the potassium concentration of the serum.

The calcium concentration of saliva was almost always higher than that of serum, and usually ranged from 6 to 8 milli-equivalents per liter. Occasionally initial values were as high as 10 milli-equivalents per liter.

The pH remained quite constant throughout control experiments. While parallel fluctuations of total carbon dioxide and pH in the experiments reported in Tables I and III indicate the possibility of a relationship between these two factors, Table V shows wide discrepancies. Furthermore, saliva from different animals may have the same pH but differ much in total carbon dioxide concentration. For example, Table III shows a pH of 7.9 accompanied by a bicarbonate concentration of 85.2 milli-equivalents per liter, and in Table V an identical pH value is found with a saliva which contained only 56.6 milli-equivalents. While the bicarbonate concentration undoubtedly is concerned with the pH, other factors must enter also. No correlation, such as Ball (14) reported for pancreatic juice, between rate of secretion and pH could be discerned in these experiments.

The secretion of sodium and chloride seems to be so controlled by the gland that the concentrations of these substances show little change on the intravenous injection of sufficient 10 per cent sodium chloride to cause a marked rise in the sodium and chloride concentration of the serum (see Table II).

## Inorganic Composition of Saliva

TABLE II  
*Injection of Sodium Chloride and Calcium Chloride*  
 Dog 6, male, weight 20.0 kilos.

Sample No. ....	Blood serum			Right parotid saliva				
	1	2	3	1	2	3	4	5
Time of collection	10.50 a.m.	11.40 a.m.	2.15 p.m.	10.45-	11.35-	12.00 m.-	1.42-	2.07-
Volume, cc. ....	40*	40*	40*	11.04 a.m.	11.58 a.m.	12.45 p.m.	2.05 p.m.	2.25 p.m.
Rate, cc. per kg. per hr. ....				12.0	20.0	15.0	20.0	10.0
Solids, per cent....				1.9	2.6	1.0	2.6	1.7
Ash " "				1.420	0.960	0.809	0.805	0.775
HCO <sub>3</sub> , m.-eq. per l.	23.9	22.5	21.5	0.750	0.783	0.723	0.726	0.683
Cl	" " 107.9	124.9	130.4	51.7	47.8		47.6	
Na	" " 136.5	150.4	156.9	84.2	97.9	80.4	75.3	
K	" " 5.5	4.8	5.4	11.2	9.8	9.4	13.7	13.3
Ca	" " 6.4	5.8	12.5	9.0	8.7	7.6	17.2	14.6

Pilocarpine was given intravenously at 10.45, 11.35 a.m., and 1.50 p.m. 92 cc. of 10 per cent NaCl were given intravenously from 11.16 to 11.32 a.m. 25 cc. of 10 per cent CaCl<sub>2</sub> were given intravenously from 1.15 to 1.20 p.m.

\* Amount of whole blood.

TABLE III  
*Injection of Sodium Carbonate*

Dog 13, female, weight 20.5 kilos.

Sample No. ....	Blood serum			Right parotid saliva					
	1	2	3	4	1	2	3	4	5
Time of collection..	11.15 a.m.	12.19 p.m.	1.27 p.m.	3.30 p.m.	10.21-	11.02-	12.23-	1.27-	2.16-
Volume, cc. ....	35*	15*	35*	50*	11.02 a.m.	11.37 a.m.	1.22 p.m.	2.12 p.m.	3.30 p.m.
Rate, cc. per kg. per hr. ....	.....	.....	.....	.....	.....	.....	.....	.....	18.0
Solids, per cent....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Ash	" "	" "	" "	" "	0.590	0.344	0.599	0.836	0.914
pH.....	.....	.....	.....	.....	0.388	0.254	0.512	0.736	0.763
HCO <sub>3</sub> , m.-eq. per l.	23.1	30.9	48.7	38.8	7.6	7.5	7.7	7.8	7.9
Cl	" "	113.5	112.1	108.4	111.2	37.5	26.5	18.6	85.2
Na	" "	148.3	140.4	171.4	163.5	48.8	48.2	76.1	56.3
K	" "	3.7	3.3	3.0	10.2	6.1	6.5	5.2	122.6
Ca	" "	5.8	5.1	5.7	5.8	5.0	6.2	6.4	5.9

Pilocarpine was given intravenously at 10.08, 11.18 a.m., 12.47, 1.51, and 2.30 p.m. 156 cc. of 10 per cent Na<sub>2</sub>CO<sub>3</sub> were given intravenously from 11.54 a.m. to 1.33 p.m.

\* Amount of whole blood.

The results of sodium carbonate injections stand in striking contrast to the above. When the concentrations of sodium and total carbon dioxide in the serum were increased by intravenous injections of 10 per cent sodium carbonate, a prompt increase of both the sodium and total carbon dioxide of the saliva occurred (Table III). As one would expect, the pH of the saliva was increased following the injections.

The ratio of the concentration of the total carbon dioxide of the serum to that of the saliva may differ from animal to animal but

Dog 10, male, weight 17.4 kilos.

Sample No.....	Blood serum		
	1	2	3
Time of collection.....	10.20 a.m.	1.11 a.m.	2.00 p.m.
Volume, cc.....	40*	40*	80*
Rate, cc. per kg. per hr.....			
Solids, per cent.....			
Ash " "			
HCO <sub>3</sub> , m.-eq. per l.....	26.8	23.8	22.6
Cl " " "	108.3	109.8	114.3
Na " " "	130.3	142.8	146.1
K " " "	3.3	5.1	5.9
Ca " " "	6.0		6.4

Pilocarpine was given intravenously at 10.16, 11.48 a.m., 12.34, and 1.32 p.m.

\* Amount of whole blood.

tends to remain the same for each animal after the injection. The same cannot be said of the chloride ratio, and the sodium ratio exhibited constancy only in the sodium carbonate experiments.

Following the injection of sufficient calcium chloride solution to more than double the calcium concentration of the serum, a large increase in the calcium of the saliva occurred (Table II). The ratio of the concentrations of the calcium of the serum to that of the saliva usually tended to increase slightly.

The injections of potassium salt solutions were made intra-arterially to lessen the toxic effect. The animals seemed to be able

to withstand the injection of potassium as the carbonate better than as the chloride. Because control experiments indicated that potassium concentrations were more uniform some time after the experiment was begun, the potassium salt injections were delayed until three or four control samples had been collected (see Table IV). Although a rise in the concentration of the potassium of the saliva followed each injection, the variations are of doubtful significance. On the supposition that potassium as the carbonate might exhibit a behavior similar to that of sodium carbonate, intraarterial

Left parotid saliva

2	3	4	5	6	7
10.26-	10.42-	10.59-	11.27 a.m.-	12.31-	1.14-
10.42 a.m.	10.59 a.m.	11.21 a.m.	12.17 p.m.	1.08 p.m.	2.10 p.m.
5.5	5.5	8.5	28.0	15.5	18.0
1.2	1.0	1.4	1.7	1.4	1.0
0.860	0.750	0.699	1.157	1.016	0.847
0.650	0.535	0.493	0.673	0.672	0.619
	55.6		64.7		82.0
60.6	49.2	48.4	53.1	37.4	26.5
103.5	84.3	71.3	98.0	100.0	85.3
5.3	4.3	5.4	9.0	11.4	12.3
5.8	5.4	7.0	8.2	7.4	8.1

per cent KCl were given intraarterially from 12.25 to 12.56 p.m.

injections of 10 per cent potassium carbonate were made. The results obtained indicated no such parallelism. In fact, values for the potassium content of saliva, obtained in other experiments in which no potassium was injected, were often just as high or higher.

When hydrochloric acid was injected (see Table V) a sharp drop in the total carbon dioxide content of the serum followed, as was to be expected. Contrary to the report of Hug and Marenzi (15) no regular fall in the carbon dioxide content of the saliva was noticed. However, it was found, as the above investigators re-

TABLE V  
*Injection of Hydrochloric Acid*

Dog 22, female, weight 8.7 kilos.

Sample No.....	1	2	3	Blood serum	Saliva			
					Gland	1	2	3
Time of collection.....	10.45 a.m.	11.17 a.m.	12.35 p.m.		10.45 a.m.	11.12-	11.00-	11.30 a.m.
Volume, cc.....	40*	40*	80*		R.	9.0	9.5	12.00 m.
Rate, cc. per kg. per hr.....					L.	10.0	8.5	12.5
Solids, per cent.....	9.540	9.850	9.483		R.	1.9	3.2	12.5
Ash " "	0.833	0.883	0.843		L.	2.1	2.9	4.0
pH.....					R.	0.977	0.770	0.870
HCO <sub>3</sub> , m-equiv. per l.....	19.3	14.6	15.4		L.	1.030	0.750	0.836
Cl	" "	111.8	112.3		R.	0.587	0.530	0.940
Na	" "	130.5	135.7		L.	0.550	0.490	0.586
					R.	7.7	7.7	8.0
					L.	7.7	7.8	8.0
					R.	54.8	47.4	47.6
					L.	51.7	44.3	46.3
					R.	51.8	54.8	55.3
					L.	56.9	53.5	55.8
					R.	77.3	69.1	77.7
					L.	83.6	70.3	79.8
								76.7

Pilocarpine was given intraperitoneally at 10.00, 10.55, 11.37 a.m., 12.05, and 12.22 p.m. 18 cc. of 1.0 N HCl were injected in the femoral artery from 10.58 to 11.13 a.m. 9 cc. of 1.0 N HCl were injected in the femoral artery from 12.11 to 12.19 p.m. Saliva ceased flowing at 12.15 p.m.

\* Amount of whole blood.

port, that a rise in pH occurred. No explanation is offered for this phenomenon. In several other experiments not reported in detail, the injection of acid was followed by a cessation in the flow of saliva in spite of repeated injections of pilocarpine.

Qualitative tests on the saliva showed that sulfocyanate was absent and that inorganic sulfate was present in small amounts. Additional sulfur was present in some other combination, possibly as an organic ester. This was shown by the relatively much greater precipitate of barium sulfate obtained with ashed saliva as compared with the unashed. Moreover, when the fresh saliva was allowed to stand in contact with dilute hydrochloric acid overnight, a large increase in ionic sulfate was noted. Quantitative analyses of several samples of parotid saliva showed magnesium 0.6 to 1.2 milli-equivalents per liter and phosphate 0.4 to 0.7 milli-equivalents per liter.

#### DISCUSSION

The saliva secreted by the parotid gland of the dog, under pilocarpine stimulation, is markedly different from the serum. A comparison of the two fluids is shown in Table VI. Bicarbonate, potassium, and calcium are usually present in higher concentration in the saliva than in the serum; chloride and sodium are lower in saliva. It is thus evident that saliva is not a simple transudate or dialysate. There appears to be no tendency toward a diminution of the efficiency of the controlling mechanism with regard to any constituent studied after injections which alter the composition of the serum considerably.

The pancreas produces an alkaline secretion of quite different composition from the saliva. Ball showed (16), in this laboratory, that the sodium and potassium apparently filter through the pancreas because he found the concentrations are the same in the water of both the pancreatic juice and serum. The calcium, magnesium, and phosphate are far below the concentrations in the serum. The chloride and bicarbonate vary with the rate of flow of the pancreatic juice, the bicarbonate rising and the chloride falling as the rate increases. The variations of these two ions appear to control the pH of the secreted juice. Injections of inorganic salts do not alter appreciably the above relations.

Ball found that pancreatic juice and serum had the same osmotic

pressure. No such equality appears to exist between saliva and serum. Nolf (17) reports freezing point depressions for the submaxillary saliva of the dog of  $-0.193^{\circ}$  to  $-0.396^{\circ}$  when obtained by chorda tympani stimulation, and  $-0.109^{\circ}$  to  $-0.266^{\circ}$  for the spontaneously secreted saliva. The serum had a freezing point depression of  $-0.560^{\circ}$ . The freezing point depression for dog parotid saliva calculated on the basis of the inorganic concentration as shown in Table VI is about  $-0.48^{\circ}$ .

TABLE VI

*Comparative Composition of Serum, Parotid Saliva, Pancreatic Juice, Dialysates, and Transudates of the Dog*

The values are expressed as milli-equivalents per liter.

	Serum	Parotid saliva	Pancreatic juice	Dialysate	Transudate
HCO <sub>3</sub> .....	25.3	55.1	100.0	22.2	26.1
Cl.....	110.1	81.7	51.0	122.8	126.0
Na.....	135.2	106.0	158.0	147.0	149.7
K.....	4.5	10.1	6.3	3.8	5.0
Ca.....	5.9	7.7	1.0	3.2	3.5

The figures given for parotid saliva are average values taken from control data. The values for pancreatic juice are from the work of Ball (18). The figures for dialysates and transudates are taken from the papers of Greene and Powell (18) and Greene, Bollman, Keith, and Wakefield (19) respectively.

## SUMMARY

1. Saliva obtained from the parotid gland of the dog by means of pilocarpine stimulation was analyzed for total solids, pH, total carbon dioxide, chloride, sodium, potassium, and calcium.
2. Total carbon dioxide, calcium, and potassium were found to be present in the saliva in greater concentration than in the blood serum, while sodium and chloride concentrations were lower in saliva than in the serum.
3. The intravenous injections of solutions of calcium chloride and of sodium carbonate in quantities sufficient markedly to increase the concentrations of their respective ions in the blood were followed by increases in the concentration of these substances in the saliva.

4. The injection of concentrated solutions of sodium chloride, potassium chloride, and potassium carbonate into the blood stream did not materially affect the composition of the saliva.

5. The intravenous injections of either hydrochloric acid or of sodium carbonate resulted in an increase of the pH of the saliva.

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## **IRRADIATED MILK: THE AMOUNT OF VITAMIN D AND ITS RATE OF FORMATION**

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Various studies have been made on the character of the photochemical reactions involved in the formation of vitamin D by ultra-violet rays. The majority of these investigations has been directed to the determination of the specific wave-lengths concerned and the quantum efficiency of the reaction. Substantial contribution to our knowledge of the principles which are operative in the formation of vitamin D from pure sterols has been made by these studies, but as yet there are few or no data which demonstrate the degree to which these principles may be applicable to food substances of heterogeneous composition. Obviously, investigations of such a character are not applicable to all food products, but in view of the results which have been obtained from standardized methods for the irradiation of milk with controlled sources of radiation, it is now possible to present data showing the applicability of certain principles involved in the activation of this product.

It has been found impossible to apply directly the available quantitative data on the energy required for activating pure sterols to the process of activating milk. Irrespective of the speculative reasons which may be advanced in explanation of the greater amount of energy required to activate milk, one obvious reason lies in the fact that it is of heterogeneous composition and various constituents, particularly the protein content, act as a screen which prevents penetration of the activating rays to any appreciable depth. Unpublished data show that these rays do not penetrate milk beyond a depth of 0.1 mm.

*Methods*

A series of experiments has been carried out wherein a variety of sources of radiation of known spectral energy distribution and intensity was used to irradiate milk under controlled conditions. The energy required for producing different degrees of antirachitic potency has already been reported (1). Since the kind and amount of radiation used, the amount of milk exposed, the conditions of exposure, and the resulting antirachitic properties were all definitely determined, the data can be used to calculate the efficiency of the reaction and to make other significant calculations. The milk used contained 1.2 per cent of butter fat.

The sources of radiation were carbon arcs burning between vertical electrodes and quartz mercury vapor lamps of the Hanovia type. Four arcs were placed 61 cm. from two vertical flowing milk films whose dimensions were 163  $\times$  853 cm. The thickness of the milk films was maintained at 0.4  $\pm$  0.03 mm. Uniform conditions of agitation were provided which permitted a constant succession of fresh surface exposures.

Large chromium-plated reflectors were placed behind the arcs in order to supplement the direct rays. These reflectors were designed to provide a uniform intensity of the combined direct and reflected rays striking the milk surface. The radiation from the various arcs was measured and expressed in terms of ergs per second falling on a sq. mm. 61 cm. horizontally from the arc. These data are shown in Table I which also includes the equivalent quanta for each source of radiation. Our calculations will be based on the total energy between 2000 and 3000 Å. This appears permissible in view of the work of Kon, Daniels, and Steenbock (2), Bourdillon and collaborators (3), and Marshall and Knudson (4), wherein it is indicated that the activation of ergosterol is not dependent on a specific wave-length within this range. It has been determined that wave-lengths above 3000 Å. applied to milk under the conditions of these experiments are of but slight practical value in contributing to the antirachitic potency obtained.

*Calculations from Experimental Data*

The method of calculating the total energy (2000 to 3000 Å.) incident to the milk is illustrated by the following example.

The rays can be considered to emanate in all directions from the

arc, which will be considered as a point source of light. The space around the arc will be considered as two hemispheres. The hemisphere toward the milk receives the direct radiation from the arc and the radiation from the opposite hemisphere is returned, in part, to the milk by the reflector. By calculating the area of the spherical polygon which is a projection of the rectangular sheet of milk, it is found that the milk subtends 49 per cent of the solid angle about the arc in the near hemisphere. Similarly, by calculating the spherical area of the projection of the reflector we find that it subtends 70 per cent of the solid angle about the arc in the far hemisphere. The reflecting surface of the polished chromium reflects substantially 60 per cent of the radiation below 3000 Å. Hence this hemisphere contributes the equivalent of 42 per cent of its solid angle. The total radiation received by the milk is therefore one-half (49 + 42 per cent) or 45.5 per cent of the total radiation from the arc. Although it is known that milk reflects certain radiations, experimental evidence shows that the energy below 3000 Å. reflected under the conditions of our experiments was only slight and can be disregarded in these calculations.

The unit solid angle at a distance of 61 cm. contains  $610 \times 610$  or 372,100 sq. mm.; 45.5 per cent of the 12.57 such areas around the theoretical point source of radiation gives 2,124,000 sq. mm. Because of slight shadows cast by the supporting mechanism, an area slightly smaller than this is actually effective. For ease of calculation, it may be assumed that 2,000,000 times the amount of radiation that falls on 1 sq. mm. 61 cm. horizontally from the arc reaches the milk. Since four arcs operating under identical conditions were used, the total figure is 8,000,000. Minor irregularities in the contour of the reflector necessitated by mechanical limitations and such assumptions as are indicated above may be responsible for an error of 5 to 10 per cent in this figure. However, this degree of error will not affect the order of magnitude of the efficiency of the reaction with which we are concerned.

Since the figure derived as above is a constant for the conditions prevailing in these experiments, and since the energy from any given source had already been evaluated, the total energy incident on the milk is readily determined. In this illustrative example we shall use 128.52 ergs per second per sq. mm. (Table I)  $\times$  8,000,000. This makes a total of  $1028 \times 10^6$  ergs per second applied to the

## Vitamin D in Irradiated Milk

TABLE I  
Character of Radiation between 2000 and 3000 Å. Used for Irradiating Milk

Group No.	Sample No.	Arc	Energy per sec. per sq. mm. at normal incidence to milk at 61 cm. from arc						Total 2000-3000 Å.		
			2000-2200 Å.		2200-2400 Å.		2400-2600 Å.				
			ergs ( $\times 10^{15}$ )	quanta ( $\times 10^{15}$ )	ergs ( $\times 10^{15}$ )	quanta ( $\times 10^{15}$ )	ergs ( $\times 10^{15}$ )	quanta ( $\times 10^{15}$ )			
I-AC-60	5-8	C carbon	7.56	0.808	19.44	2.275	34.56	4.400	31.32	4.310	
I-AC-80	9-12	" "	12.98	1.390	33.48	3.920	55.08	7.020	45.36	6.230	
II-AS-60	65-68	Sunshine carbon	0.76	0.081	3.24	0.379	7.56	0.963	8.10	1.114	
II-AS-80	69-72	" "	0.97	0.103	4.32	0.505	10.80	1.378	10.80	1.487	
III-AMG-60	125-128	Magnesium carbon	1.66	0.177	4.44	0.520	11.95	1.522	38.40	5.280	
III-AMG-80	129-132	" "	2.78	0.297	7.43	0.870	19.60	2.500	60.00	8.260	
IV-AHG	181-184	Mercury vapor*	4.43	0.475	10.65	1.248	24.10	3.070	19.60	2.700	

\* Values calculated at 30.5 cm. instead of 61 cm.

milk. With a rate of exposure of 1042 cc. per second for this particular case, the energy (2000 to 3000 Å.) applied per cc. is  $\frac{1028 \times 10^6}{1042}$  or  $989 \times 10^3$  ergs.

Calculations similar to the above were made for each source of radiation operating under controlled conditions and emitting energy values, as shown in Table I. The maintenance of uniform conditions under which the milk was exposed to each radiation permits a comparable evaluation of the various sources and energy applications, as shown in Table II. In order that variations in the amount of energy might be obtained, the milk was exposed for different periods. A single time unit was 8 seconds. Six time units or 48 seconds were the maximum period for which the milk was exposed in any of these experiments.

Immediately following irradiation the milk was dried by the Just process and hermetically sealed in inert gas.

The vitamin D potency of the experimental samples was determined with white rats kept on the Steenbock and Black (5) Ration 2965. Animals selected from the stock colony at 28 to 30 days of age were given the rachitogenic ration for 21 days. After this period the reconstituted experimental milks were fed in 0.5 to 10 cc. quantities daily for a period of 10 days. At the end of this period, the microscopic line test was made according to the usual procedure. Since it was desired to determine the amount of each of the milks required to give a minimum detectable antirachitic effect, the +1 degree of calcification (6, 7) was used as the standard of evaluation. The results of these assays are based on groups of animals containing from four to ten individuals. While variation in the results from individual animals is a common experience when this method of assay is used, observations based on the determination of the antirachitic potency of numerous samples of activated milk warrant the belief that the amount of milk recorded in each instance as necessary to produce the +1 degree of calcification is correct to within 10 per cent. When the amount of milk required to produce a uniform minimum effect is known and the energy per cc. applied, the calculation of various relationships is possible.

An illustrative calculation will be shown by using the specimen sample selected heretofore. By reference to Table I it will be

noted that a total of 128.52 ergs is equivalent to  $17.063 \times 10^{12}$  quanta. The number of quanta per erg is therefore  $\frac{17.063 \times 10^{12}}{128.52}$  or  $1325 \times 10^8$ . Since the total number of ergs applied per cc. of milk in this case is  $989 \times 10^8$ , the total quanta would be  $989 \times 10^8 \times 1325 \times 10^8$ , or  $1323 \times 10^{16}$ . The total quanta required to produce the +1 line test in this case are therefore  $5949 \times 10^{15}$ , since 45 cc. were used.

If it is assumed that the +1 degree of calcification selected as the standard of evaluation is equivalent to the minimum antirachitic effect obtained by  $0.03 \gamma$  (0.00003 mg.) of the vitamin D isolated by Windaus (8), the molecular concentration of the vitamin in the treated milk can be calculated, since there seems to be definite evidence showing that its molecular weight is the same as that of ergosterol ( $C_{27}H_{42}O$ ) (3, 4, 8, 9). On the basis of these assumptions, each quantity of milk required to give a +1 line test must have contained the equivalent of  $0.03 \gamma$  or  $4.5 \times 10^{13}$  molecules of vitamin D.

#### DISCUSSION

Interpretation of the data shown in Table II is facilitated by Charts 1 and 2. In Chart 1 the amount of the different milks required to give the +1 degree of calcification is plotted against the ergs per cc. applied to the milk. This is a composite curve constructed from the data obtained from all sources of radiation. It indicates the relative efficiency of utilization of the energy for vitamin D formation in milk irradiated under the conditions of these tests. It will be noted that the amount of vitamin D formed shows evidence of a definite relationship to the amount of energy applied only during the very early stages of the reaction. It is believed that this result tends to parallel the observations of others (3, 4, 10) concerning the character of the photochemical reactions involved in the activation of pure ergosterol, wherein it has been shown that the rate of formation of vitamin D is relatively rapid during the early stages of the reaction, and that, up to certain limits, the amount of vitamin D formed is dependent upon the energy input.

The fact that different amounts of milk were required to obtain the same degree of calcification might be construed as introducing

an element of variability affecting the reliability of the relationships shown. This possibility cannot be wholly ignored. However, since we are dealing with milk as an entity, the relationships observed will be confined to the product in question without attempts to compare them unjustifiably with those obtained from pure sterols. It may be mentioned, however, that adjustments in the Ca:P ratio equivalent to the feeding of as high as 300 cc. of milk over a period of 10 days did not alter the relationships attributed to vitamin D. It would appear therefore that variations in the Ca:P ratio, such as existed in the experimental diets when var-

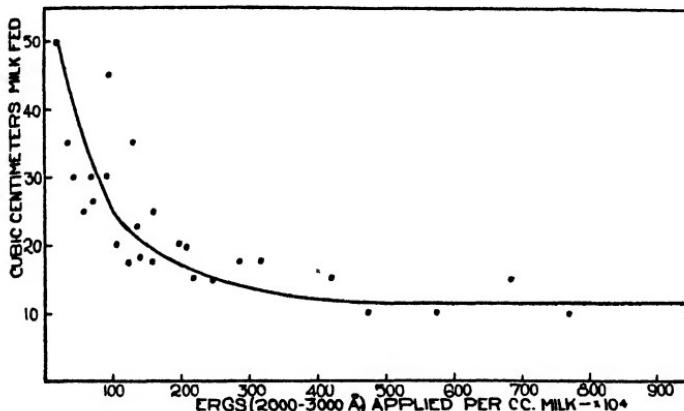


CHART 1. The relationship between the energy applied and the amount of milk required to produce the minimum detectable antirachitic effect.

iable amounts of milk were fed, can be disregarded as having materially affected the results.

Non-irradiated milk of the same composition as that used in these experiments has a small but measurable vitamin D content. Various determinations show that from 150 to 200 cc. fed over a period of 10 days are required to give the +1 line test. It is possible, therefore, that the results from the slightly activated milks which required the feeding of from 40 to 50 cc. were somewhat influenced by the inherent vitamin D content of the non-irradiated product. It is improbable that the results from the more highly activated products were affected to any measurable extent.

In order that the utilization of the energy and the formation of vitamin D in milk may be shown more clearly, the curves in Chart 2 have been constructed by plotting the number of molecules resulting from 1 quantum of energy (quantum efficiency) against the number of quanta applied per cc. Curves have been prepared for each source of radiation used, thus permitting an examination of the relative merits of these sources, and also furnishing further evidence regarding the rate of formation of vitamin D in milk and the efficiency of the reaction.

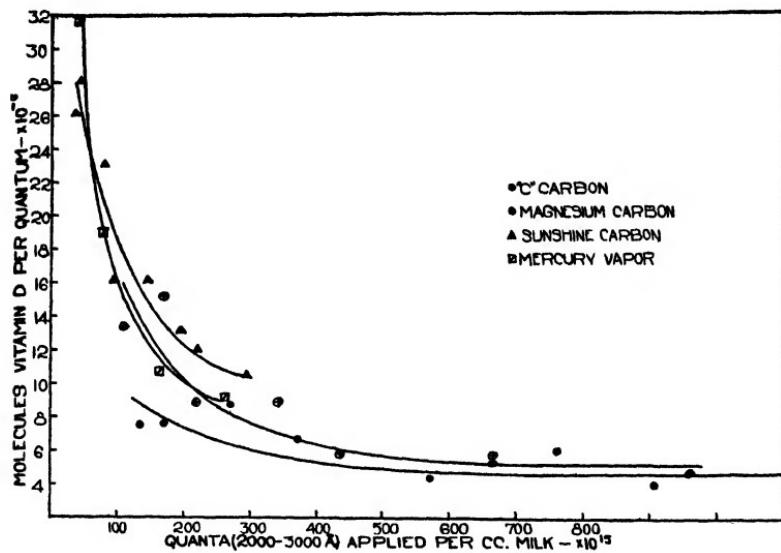


CHART 2. The rate of formation of vitamin D in milk by different sources of radiation.

It is to be noted that the quantum efficiency is relatively high during the early stages of the reaction, and with the lower amounts of energy, which in these experiments represent an exposure period of only a few seconds. During this period a direct relationship between the amount of energy applied and the amount of vitamin D formed is indicated. As the energy is increased beyond a certain point, which is in the neighborhood of  $2000 \times 10^{14}$  to  $3000 \times 10^{14}$  quanta per cc. little or no additional vitamin D is formed, or, if formed, it is destroyed at the same rate. Such a phenome-

non has been observed in studies on the activation of pure ergosterol (4).

TABLE II  
*Relation of Amount of Energy Applied to Milk and Vitamin D Concentration*

Group No.	Sample No.	Arc	Time sec.	Total ergs per cc. milk ( $\times 10^6$ )	Total quanta per cc. milk ( $\times 10^6$ )	Total milk fed cc.	Total quanta fed ( $\times 10^6$ )	Vitamin D per cc. mols.	Quanta applied per molecule vitamin D ( $\times 10^6$ )	Vitamin D per quanta ( $\times 10^{-4}$ ) mols.
I-AC-60	5	C carbon	8	989	1323	45	5949	100	13.20	7.6
	6		16	2016	2676	20	5350	225	11.80	8.4
	7		32	4292	5700	15	8550	300	18.10	5.5
	8		48	6780	9012	15	13500	300	35.00	3.3
I-AC-80	9	C carbon	8	1304	1725	35	6040	128	13.40	7.7
	10		16	2836	3730	17.5	6527	256	14.50	6.8
	11		32	5748	7560	10	7560	450	16.80	6.0
	12		48	9486	12480	10	12480	450	27.70	3.6
II-AS-60	65	Sunshine carbon	8	252	345	50	1725	90	3.84	26.3
	66		16	528	762	25	1904	180	4.24	23.5
	67		32	1018	1392	20	2784	225	6.15	16.2
	68		48	1557	2130	17.5	3730	256	8.29	12.0
II-AS-80	69	Sunshine carbon	8	339	459	35	1608	128	3.57	28.0
	70		16	694	938	30	2814	150	6.25	16.0
	71		32	1412	1912	18	3440	250	7.65	13.1
	72		48	2112	2862	15	4293	300	9.54	10.4
III-AMG-60	125	Magne- sium car- bon	8	796	1105	30	3315	150	7.40	13.5
	126		16	1548	2150	25	5375	180	11.90	8.4
	127		32	3124	4340	17.5	7600	257	16.90	5.9
	128		48	4740	6588	12.5	8235	360	18.30	5.4
III-AMG-80	129	Magne- sium car- bon	8	1225	1700	17.5	2975	357	6.60	15.1
	130		16	2430	3374	15	5050	300	11.20	8.9
	131		32	4740	6580	10	6580	450	14.60	6.9
	132		48	7670	10620	10	10620	450	23.60	4.2
IV-AHG	181	Mercury vapor	8	342	459	30	1377	150	3.03	33.0
	182		16	650	870	26.7	2325	168	5.17	19.3
	183		32	1328	1784	23.3	4160	193	9.25	10.8
	184		48	1938	2598	20	5196	225	11.50	8.7

Table II shows that the length of time required for the application of the quanta necessary to cause the maximum antirachitic potency depends upon the source of radiation. Even with the lowest energy applications from the "C" and magnesium carbon

arc sources, substantially the maximum potency was obtained, whereas with the mercury and Sunshine carbon arc sources a similar degree of potency was not obtained until the highest amount of energy from these sources had been applied. If observations had been made on the C and magnesium carbon arc sources throughout the same energy range as observed for the mercury and Sunshine carbon arc sources, and if observations on the latter had been made throughout the full energy range observed for the former, it is probable that the general relationships indicated would have been further confirmed. However, an inspection of the data might indicate that the greatest efficiency of the reaction and the highest degree of potency are to be expected when sources of radiation are used which emit a high energy value within the shortest period of time.

If it is permissible to assume that the vitamin D of the irradiated milk causes the same physiological effects as the crystalline product obtained by Windaus (8), the concentration of the vitamin in the different milks is readily determined. Examples selected from Table II show that the maximum concentration was found in those samples which required only 10 to 15 cc. to produce the +1 degree of calcification. On the basis of our assumptions, this milk must have contained  $2 \gamma$  (0.002 mg.) to  $3 \gamma$  (0.003 mg.) of vitamin D per liter. The sample showing the minimum degree of potency required 50 cc. to produce the same effect. This milk contained  $0.6 \gamma$  (0.0006 mg.) per liter. Since it has been shown that about 175 cc. of non-irradiated milk are required to give the +1 line test, such milk contains only  $0.173 \gamma$  (0.000173 mg.) per liter. These observations show that the maximum vitamin D potency attained in the milk treated under the particular conditions of these experiments was about 12 times that of the non-irradiated product. The milk showing the least activation had about 3.5 times the antirachitic potency of the non-irradiated product.

#### SUMMARY

1. Up to certain limits the degree of antirachitic potency which can be imparted to milk bears a direct relationship to the amount of energy applied; beyond these limits additional energy does not increase the vitamin D content proportionately.
2. The rate of vitamin D formation in milk and the quantum

efficiency of the reaction are greatest during the very early stages of the process, which, under the conditions of these experiments, occurred during the first few seconds of exposure.

3. The maximum concentration of vitamin D in the milk irradiated according to these methods was found after approximately 2,500,000 ergs per cc. had been applied. Calculations indicate that this concentration is equivalent to about 0.0025 mg. per liter, or practically 12 times that of the non-irradiated milk.

4. Carbon arcs and quartz mercury vapor arcs, each with a different spectral energy distribution are capable of producing substantially the same vitamin D concentration in milk when comparable amounts of energy are applied. However, the data indicate that those sources of radiation which emit the greatest amount of energy per unit of time are preferable for the most efficient activation of milk exposed under the conditions prevailing in these experiments.

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## GLUCOSIDE FORMATION IN THE COMMONER MONOSSES

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The present communication deals with the rates of formation of the furanosides and pyranosides of the commoner monoses, as a function of their configuration. The original objective of the investigation was of a purely practical nature; namely, the desire to establish the optimal conditions for the preparation of certain furanosides. It is generally known that furanosides are formed with greater velocity than the corresponding pyranosides and methods of preparation have been based on this difference. Moreover, although it is well known that the configuration of the sugar affects the rates of glucoside formation, no comparative information of a quantitative nature has been hitherto available. Studies have now been made on several monoses as regards their furanose and pyranose formation under comparable conditions.

The data are presented in Table I and in Figs. 1 and 2. Fig. 1 is a set of graphs showing the changes in the proportions of free sugar and of furanoside and pyranoside. It will be noted that for each sugar investigated, the amount of furanoside rises to a maximum and then gradually decreases. Particular interest arises from the fact that this maximum production is not always clearly indicated by the curves (Fig. 2) of optical rotation (for example, mannose, fructose, arabinose, xylose) and yet it is at this point that the experiment should be interrupted in order to obtain the maximum yield of furanoside. In the cases of ribose and xylose, in which we were particularly interested, the proportion of furanoside approaches 100 per cent so that modification of the present experimental conditions was unnecessary. However, in the case of other sugars, it is possible that the proportion of furanoside could be appreciably increased by changes in temperature, or in

## Glucoside Formation in Monoses

TABLE I  
*Glucoside Formation at 25° from Reduction Determinations*

Sugar	Time	Co. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Corrected, per cent*			Distribution of sugar, per cent		
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	A	B	B <sub>c</sub>	Free	Glucoside	
Glucose	hrs.											γ	Normal
Glucose	0	9.47	9.84	3.17	8.86	102.4	95.5	101	98	98	101	-3	2
	1	7.07	9.68	2.38	8.72	76.8	93.8	76	96	96	76	20	4
	3	4.48	9.58	1.52	8.63	49.0	92.9	48	95	95	48	47	5
	7	2.41	9.30	0.82	8.38	26.4	90.1	26	92	92	26	66	8
	24	1.08	8.18	0.37	7.36	11.9	79.3	12	81	80	12	68	20
	48	0.95	7.46	0.32	6.72	10.3	72.2	10	74	73	10	63	27
Present				3.10	9.30								
Mannose	0	9.56	9.52	3.25	8.57	104.8	92.2	99	94	94	99	-5	6
	1	8.32	9.04	2.83	8.14	91.3	87.5	86	89	88	86	2	12
	3	6.20	8.70	2.14	7.83	69.0	84.2	65	86	85	65	20	15
	7	3.81	7.79	1.30	7.02	41.9	75.5	40	77	76	40	36	24
	24	0.32	3.62	0.11	3.26	3.6	35.0	3	36	33	3	30	67
	48	0.10	1.38	0.03	1.24	1.0	13.3	1	14	9	1	8	91
Present				3.10	9.30								
Galactose	0	7.22	7.62	3.14	3.32	101.4	107.1	101	107	108	101	7	-8
	1	5.45	6.98	2.38	3.05	76.9	98.4	75	98	98	75	23	2
	3	3.28	6.15	1.45	2.68	46.8	86.5	46	86	84	46	38	16
	7	1.45	5.07	0.66	2.22	21.3	71.6	21	72	69	21	48	31
	24	0.42	3.63	0.19	1.60	6.1	51.6	6	52	46	6	40	54
	48	0.09	2.70	0.04	1.20	1.3	38.7	1	39	31	1	30	69
Present				3.10	3.10								
Fructose	0	6.30	11.19	2.00	3.58	64.5	115.5	67	101	101	67	34	-1
	1	0.40	11.42	0.13	3.66	4.2	118.1	4	104	104	4	100	-4
	3	0.33	11.39	0.11	3.65	3.5	117.7	4	103	103	4	99	-3
	7	0.29	11.19	0.09	3.58	2.9	115.5	3	101	101	3	98	-1
	24	0.19	10.56	0.06	3.37	1.9	108.7	2	95	95	2	93	5
	48	0.30	10.64	0.10	3.40	3.2	109.6	3	96	96	3	93	4
	216	0.27	7.47	0.09	2.54	2.9	81.9	3	72	71	3	68	28
Present				3.10	3.10								

\* The letters in this column refer to the percentages defined in the text; A, before hydrolysis, B, after hydrolysis, B<sub>c</sub>, after hydrolysis corrected.

TABLE I—Concluded

Sugar	Time	Co. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Corrected, per cent*			Distribution of sugar, per cent		
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	A	B	B <sub>c</sub>	Free	Glucoside	γ
		hrs.											Normal
Rhamnose	0	9.66	9.51	3.14	8.66	100.3	92.3	105	96	96	105	-9	4
	1	8.36	9.12	2.65	8.30	84.7	88.4	89	92	91	89	2	9
	3	7.88	8.64	2.46	7.87	78.5	83.8	83	87	85	83	3	14
	7	6.08	7.57	1.89	6.89	60.4	73.4	64	76	73	64	9	27
	24	2.11	3.91	0.65	3.56	20.8	37.9	22	39	32	22	10	68
	48	0.43	1.72	0.13	1.57	4.2	16.7	4	17	8	4	4	92
	Present				3.13	9.39							
Arabinose	0	6.23	9.56	2.25	7.17	87.2	92.7	89	98	97	89	8	3
	1	3.38	8.49	1.24	6.37	48.1	82.3	49	87	82	49	33	18
	3	0.81	7.34	0.29	5.51	11.2	71.2	11	75	69	11	58	31
	7	0.13	3.75	0.05	2.81	1.9	36.3	2	38	16	2	14	84
	24	0.09	2.99	0.03	2.24	1.2	28.9	1	30	5	1	4	95
	48	0.09	2.66	0.03	2.00	1.2	25.8	1	27	1	1	0	99
	Present				2.58	7.74							
Lyxose	0	7.66	9.55	2.34	7.16	90.7	92.6	91	96	96	91	-5	4
	1	4.52	8.34	1.57	6.26	60.8	80.9	61	83	81	61	20	19
	3	1.63	7.11	0.57	5.33	22.1	68.9	22	71	68	22	46	32
	7	0.32	5.43	0.11	4.07	4.3	52.6	4	54	49	4	45	51
	24	0.14	2.17	0.05	1.63	1.9	21.1	2	22	13	2	11	87
	48	0.11	1.05	0.04	0.79	1.6	10.2	2	11	1	2	-1	99
	Present				2.58	7.74							
Ribose	0	6.35	9.75	2.47	7.31	95.8	94.5	93	97	96	93	3	4
	1	0.08	9.68	0.03	7.26	1.2	93.9	1	96	94	1	93	6
	3	0.08	9.73	0.03	7.30	1.2	94.3	1	96	94	1	93	6
	7	0.09	9.32	0.04	6.99	1.6	90.3	2	92	88	2	86	12
	24	0.09	8.84	0.04	6.63	1.6	85.6	2	87	81	2	79	19
	48	0.12	7.73	0.05	5.80	1.9	74.9	2	76	65	2	63	35
	Present				2.58	7.74							
Xylose	0	7.50	9.64	2.49	7.23	96.6	93.3	98	95	94	98	-4	6
	1	2.00	9.70	0.64	7.28	24.8	94.1	25	96	95	25	70	5
	3	0.43	9.51	0.14	7.13	5.4	92.1	5	94	93	5	88	7
	7	0.36	9.29	0.12	6.97	4.6	90.1	5	92	91	5	86	9
	24	0.24	7.78	0.08	5.84	3.1	75.3	3	78	74	3	71	26
	48	0.27	6.68	0.09	5.01	3.5	64.6	3	66	61	3	58	39
	Present				2.58	7.74							

concentration of sugar or catalyst. The relative furanoside rates for the different sugars may be obtained from the slopes of the curves at zero time since the whole of the sugar is then available for furanoside (and pyranoside) formation. Arranged in the order

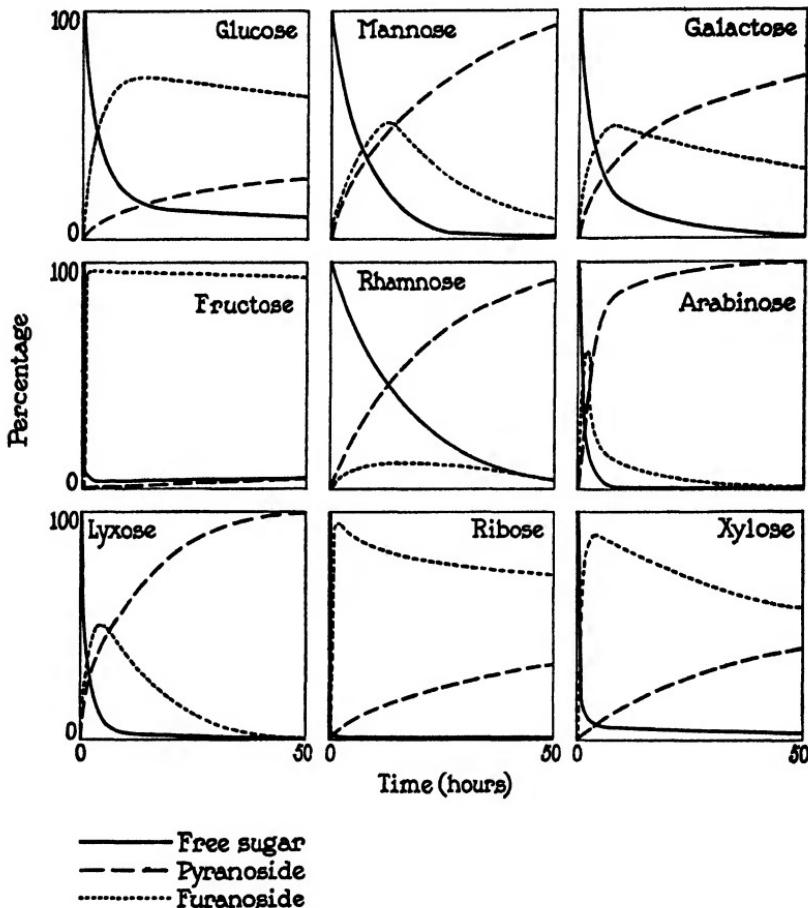


FIG. 1. Percentages of free sugar, furanoside, and pyranoside during glucoside formation at 25° in methyl alcohol containing 0.5 per cent hydrogen chloride.

of decreasing velocities of furanoside formation the sequence is fructose — ribose and xylose — galactose, glucose, and lyxose—mannose — rhamnose.

The curve of pyranoside formation offers difficulties of interpretation for the reason that the pyranosides are formed not only from the reducing sugar, but also by transformation of the furanosides. Consequently, the rate of formation is affected both directly by the configuration of the sugar and indirectly by the effect of configuration on the formation and transformation of the furanosides. The direct effect may be obtained from the slopes at zero time of the pyranoside curves (Fig. 1), and the sugars may thus be arranged in order of decreasing pyranoside rate in the series: arabinose — lyxose — mannose and rhamnose — galactose — xylose — glucose and ribose — fructose. The indirect formation is particularly noticeable in the cases of ribose and xylose. In these sugars the rate of pyranoside formation may be determined entirely by the rates of transformation of the furanosides inasmuch as after a very short interval almost no free sugar remains and yet pyranoside formation continues to take place.

*Method of Determining the Proportion of Furanosides and Pyranosides*—For purposes of estimation, furanosides and pyranosides may be differentiated by two properties, first, their optical rotation, and second, their rates of hydrolysis with dilute mineral acids. The optical method can be applied with accuracy only in a system of two components, for in a system of three or more components a knowledge of the specific rotations is not sufficient for the determination of the proportions of each. On the other hand, the character of the rotation curve may, in some cases, indicate the formation of only one or of more than one ring type of glucoside. In other instances, the curves fail to reveal the formation of two glucosides even when such formation is demonstrable by other methods. For these reasons the method of hydrolysis was resorted to. The principle of the method is very simple; namely, mild conditions suffice for the hydrolysis of furanosides whereas more drastic treatment is required for that of pyranosides.

If conditions could be found which would leave the pyranoside intact and yet would lead to complete hydrolysis of the furanoside, then  $A$ , the percentage of reducing sugar before such hydrolysis, and  $B$ , the percentage of reducing sugar after hydrolysis, are the only data which would be required.  $A$ , then, is the percentage of unreacted sugar at the particular moment,  $B - A$  is the corresponding percentage of sugar present as furanoside, and  $100 - B$

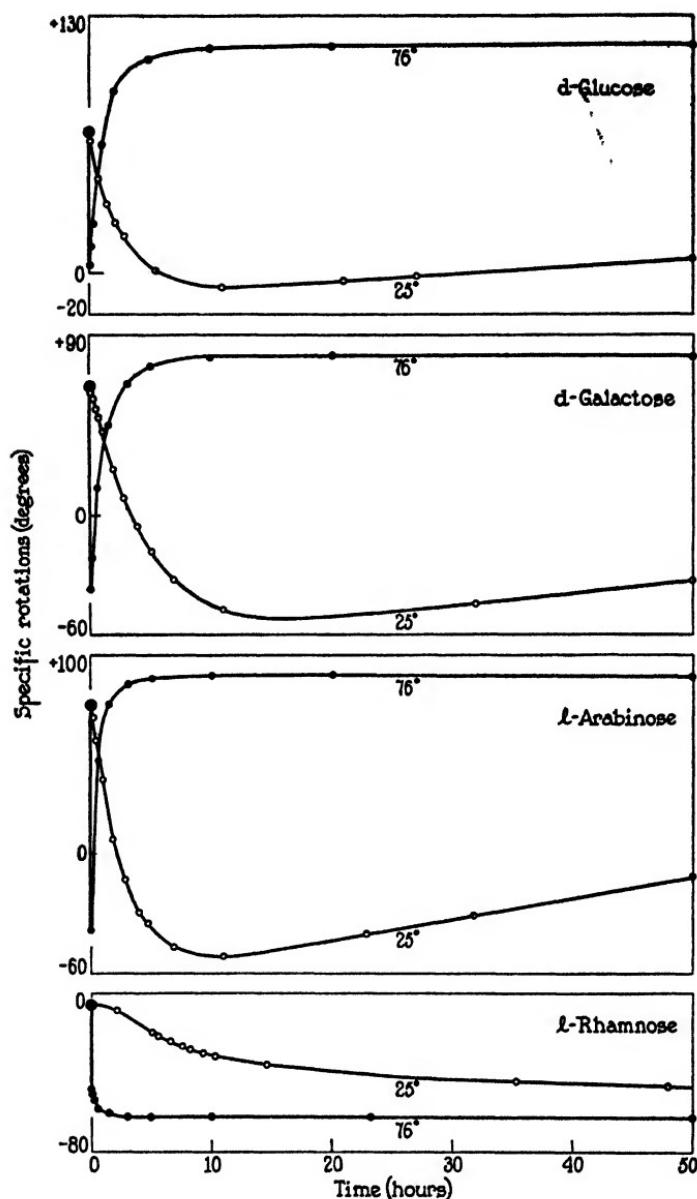


FIG. 2, a

FIGS. 2, a AND 2, b. Specific rotations during glucoside formation in methyl alcohol containing 0.5 per cent hydrogen chloride.

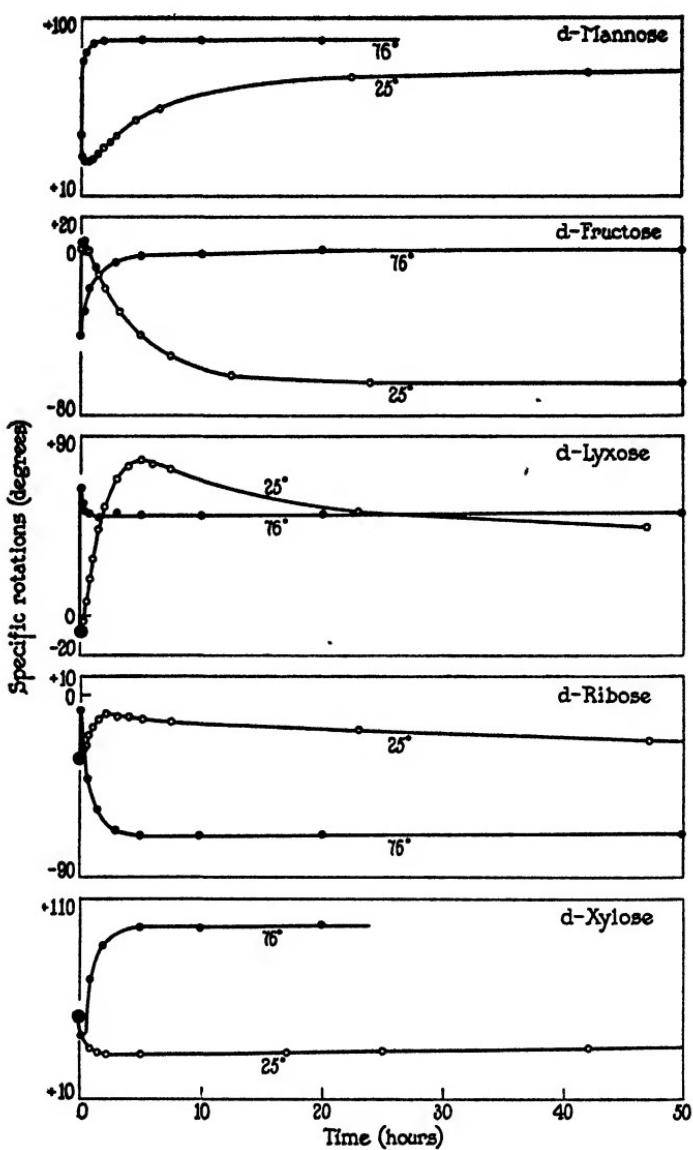


FIG. 2, b

is the percentage of sugar present as pyranoside. Actually, however, the hydrolysis of the furanoside is probably not invari-

ably quantitative nor is the hydrolysis of the pyranoside always negligible. A corrected value for  $B$  may be calculated by using the formula,  $B_c = \frac{100(B - P)}{100 - P}$ , which is derived on the assumption that  $P$ , the percentage hydrolysis of the pyranoside, is constant throughout the experiment, or in other words, that the amount of such hydrolysis in any sample is proportional only to the amount of pyranoside present in that sample. In actual fact  $P$  was determined by hydrolyzing samples which had reached optical equilibrium and hence probable equilibrium of  $\alpha$  and  $\beta$  forms of the pyranoside. The proportion of  $\alpha$  to  $\beta$  does not, however, remain constant during the experiment so that even the corrected values  $B_c$  are still generally too large. However, this error is at least in part compensated by the incomplete hydrolysis of the furanoside mentioned above. *It must be emphasized that the values obtained from the hydrolysis data are a satisfactory first approximation, permitting the determination of the time of maximum furanoside formation, and illustrating the differences in the rates of formation of furanosides and pyranosides as a function of the configuration of the sugar.*

In connection with the corrections which were used in the work, it may be mentioned that small deviations from the accepted values were found for the reducing power of our samples of sugars, particularly after they had been subjected to treatment with acid under conditions used for glucoside hydrolysis. For this reason the reducing value was determined for each sugar, both before and after acid treatment, and the factors thus obtained were used in making the calculations.

#### EXPERIMENTAL

##### *Materials*

With the exception of the glucose, which was an anhydrous, reagent grade product, all of the sugars used were prepared in this laboratory and were purified by crystallization. They were dried over phosphorus pentoxide to almost constant weight; the moisture was then determined (the maximum was 0.3 per cent) and the corresponding correction was applied to the weight of the samples.

*Procedure*

The calculated quantity of the dried sugar was weighed into a volumetric flask and dissolved in anhydrous methyl alcohol (reagent grade, acetone-free). The calculated volume of a standardized solution of dry hydrogen chloride in anhydrous methyl alcohol was added with a pipette, the mixture was immediately diluted to the desired volume with more anhydrous methyl alcohol and thoroughly mixed by shaking. The hydrogen chloride concentration in the glucoside mixtures was 0.5 per cent and the sugars were 0.344 molal. Experiments were made at room temperature and at 76°. In the latter case, portions of the solution were sealed in Pyrex test-tubes which were heated in a bath of boiling carbon tetrachloride for the required intervals of time, then removed and cooled immediately in ice.

*Analytical*

Samples of 5.0 cc. were removed as required, placed in a volumetric flask, and 2 cc. of a 0.4 N sodium carbonate solution (17 per cent excess over that required for the hydrochloric acid present) were added. After shaking well, the mixture was diluted almost to volume, cooled to room temperature, diluted to 10.0 cc., and well mixed. These samples were used for analysis.

For the determination of the free sugar the neutralized samples were analyzed without treatment as it was found that the small amount of methyl alcohol present had no effect on the reduction determinations. However, as it was learned from preliminary experiments that the hydrolysis of the furanosides was materially decreased in the presence of methyl alcohol, its removal prior to hydrolysis was necessary. The samples to be hydrolyzed were therefore placed in test-tubes fitted with distilling heads and the solutions were concentrated almost to dryness under diminished pressure. 3 to 4 cc. of water were then added and the evaporation was repeated. The residue was diluted to about 3½ cc. with water and 0.5 cc. of 0.4 N hydrochloric acid<sup>1</sup> was added. The tubes were stoppered with small funnels, heated in the steam bath for 10

<sup>1</sup> For certain sugars 0.2 N hydrochloric acid was used, and the heating was 5 minutes in some cases. The times and concentrations are listed in Tables IV and V.

minutes,<sup>1</sup> and then removed and cooled. The solutions were neutralized with the theoretical equivalent of 0.4 N sodium carbonate or sodium hydroxide, diluted to 5 cc., and the analyses were then performed as for the unhydrolyzed samples.

Two analytical methods were employed, the Hanes<sup>2</sup> modification of the Hagedorn-Jensen<sup>3</sup> method, and a micro modification of the Willstätter<sup>4</sup> hypoiodite method. For the former procedure 1.0 cc. portions of the neutralized samples were used and for the latter 3.0 cc. portions.

For the micro hypoiodite analyses (Procedure A) the samples were placed in large Pyrex tubes and diluted to 7½ cc. with distilled water. 1.5 cc. of 0.1 N iodine-potassium iodide solution were added and then, drop by drop, 1 cc. of 0.3 N sodium hydroxide. After standing 15 minutes at room temperature there were added 2 cc. of 0.2 N potassium iodide and 0.2 cc. of 5 N sulfuric acid, and the free iodine was titrated with N/70 sodium thiosulfate solution with starch as an indicator.

It was found that this procedure gave results which were considerably less than the theoretical in the case of those sugars which have *cis* hydroxyls on carbon atoms 2 and 3 (*i.e.*, mannose, rhamnose, lyxose, and ribose) so that for these sugars the procedure was somewhat modified. In Procedure B, 3.0 cc. of sample were diluted with water to 7½ cc. volume as before. After addition of 1.5 cc. of 0.1 N iodine-potassium iodide the tubes were placed in ice and allowed to cool for several minutes, and were kept in this ice bath except during the additions of alkali. The 0.3 N sodium hydroxide was added in small portions at 2 minute intervals, the tubes being well shaken during each addition. The amounts added were 0.2, 0.2, 0.1, 0.1, 0.1, 0.1, and 0.1 cc., a total of 0.9 cc. requiring 12 minutes for complete addition. The tubes were left in the ice bath for an additional 3 minutes and were then transferred to a beaker of water at room temperature, in which they were left for a further 15 minutes. Potassium iodide and sulfuric acid were added as before and the titration was performed similarly. By means of this modification in technique, the values found for

<sup>2</sup> Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>3</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

<sup>4</sup> Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

these abnormal sugars were increased from an average of 74.8 per cent of the theoretical to an average of 97.4 per cent.

### *Hydrolysis of Normal Glucosides*

Samples of the sugar, methyl alcohol, and hydrogen chloride solutions were heated at 76° in sealed tubes until no further change in rotation occurred, and the amount of free sugar was then de-

TABLE II  
*Stability of Normal Glucosides*

Sugar	Glucoside heating hrs.	Co. 0.01 N thiosulfate		Sugar, mg.		Sugar present, mg.	Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis		Before hydrolysis	After hydrolysis	
.									
Glucose	15	0.06	0.07	0.02	0.16	3.10	1	5	0.1 N, 10 min.
Mannose	4.5	0.06	0.47	0.02	0.16	3.10	1	5	0.1 " 10 "
Galactose	15	0.08	1.43	0.04	0.65	3.10	1	21	0.1 " 10 "
				0.73	0.34			11	0.05 " 10 "
Fructose	10	0.61	0.40	0.20	0.13	3.10	6	4	0.1 " 10 "
Rhamnose	4	0.05	1.04	0.02	0.32	3.13	1	10	0.1 " 10 "
Arabinose	10	0.14	2.24	0.05	0.82	2.58	2	32	0.1 " 10 "
				1.82	0.66			26	0.05 " 5 "
Lyxose	7	0.08	1.89	0.03	0.72	2.58	1	28	0.1 " 10 "
				0.67	0.25			10	0.05 " 5 "
Ribose	6	0.18	2.87	0.07	1.12	2.58	3	43	0.1 " 10 "
				2.04	0.80			31	0.05 " 5 "
Xylose	12	0.11	0.96	0.03	0.31	2.58	1	12	0.1 " 10 "

terminated by analysis. The amount was invariably found to be very small. Portions were freed of methyl alcohol by concentration as described above and were then hydrolyzed with hydrochloric acid of various strengths and for different periods of time. Some of the data thus obtained are given in Table II. These factors were used in calculating the corrected reducing values according to the formula on p. 706.

*Analytical Factors*

The data of Sobotka and Reiner<sup>6</sup> were used as standards for the Hanes modification of the Hagedorn-Jensen method but as they did not include lyxose<sup>6</sup> a standard solution of this sugar was prepared and the factors were determined. The results are given in Table III.

The samples of sugars used were all subjected to analysis, both before and after acid treatment, in order to obtain their exact reducing equivalent. The reducing values found by the Hagedorn-Jensen (Hanes) method are given in Table IV and those found by the hypoiodite method in Table V.<sup>7</sup> Before acid treatment the deviations from the theory were small but after treatment the deviations with the Hagedorn-Jensen method became

TABLE III

*Reducing Values by Hagedorn-Jensen (Hanes) Method. Cc. 0.01 N Thiosulfate*

Sugar, mg.....	1.0	2.0	3.0	4.0
Lyxose.....	2.83	5.81	8.73	
Celllobiose.....	2.58	5.10	7.61	10.09

quite large as will be seen from Table IV. It was for this reason that the hypoiodite oxidation was employed in the majority of cases for the samples after hydrolysis.

*Optical Rotation*

The rotations were measured on the 76° samples (previously cooled in ice) by warming them rapidly to room temperature and reading them in a 2 dm. tube with sodium D light. In the experiments conducted at room temperature samples were placed in

<sup>6</sup> Sobotka, H., and Reiner, M., *Biochem. J.*, **24**, 394 (1930).

<sup>7</sup> Celllobiose standards were also made but this sugar was not used for glucoside experiments. The data are given, however, for possible future reference.

<sup>7</sup> Celllobiose, lactose, and maltose (the last probably not quite pure) are appended for possible reference although they were not actually used in the glucoside formation experiments.

TABLE IV  
*Correction Factors for Hagedorn-Jensen (Hanes) Method*

Sugar	Present, mg.	Cc. 0.01 N thiosulfate		Sugar found, mg.		Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	
Glucose.....	3.0	9.06	10.54	3.03	3.52	101	117	0.1 N, 10 min.
Mannose.....	3.0	9.34	8.44	3.18	2.87	106	96	0.1 " 10 "
Galactose.....	3.0	7.03	6.86	3.06	2.99	102	100	0.1 " 10 "
Fructose.....	3.0	9.06	10.69	2.88	3.42	96	114	0.1 " 10 "
Rhamnose.....	3.0	8.96	8.00	2.86	2.52	95	84	0.1 " 10 "
Arabinose.....	3.0	8.22	8.74	2.93	3.11	98	104	0.05 " 5 "
Lyxose.....	3.0			9.36	3.20	100	107	0.05 " 5 "
Ribose.....	3.0	8.00	8.46	3.09	3.26	103	109	0.05 " 5 "
Xylose.....	3.0	8.94	9.92	2.97	3.29	99	110	0.1 " 10 "
Cellobiose.....	3.0			8.03	3.16	100	105	0.05 " 10 "
Lactose.....	3.0	6.60	6.75	2.88	2.95	96	98	0.05 " 5 "
Maltose.....	3.0	6.36	6.64	2.62	2.73	87	91	0.05 " 5 "

TABLE V  
*Correction Factors for Micro Hypoiodite (Willstätter) Method*

Sugar	Present, mg.	Cc. 0.01 N thiosulfate		Sugar found, mg.		Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	
Glucose.....	5.0	5.40	5.45	4.86	4.91	97.2	98.2	0.1 N, 10 min.
Mannose*.....	5.0	5.36	5.45	4.83	4.91	96.6	98.2	0.1 " 10 "
Galactose.....	5.0	5.01	5.10	4.51	4.59	90.2	91.8	0.1 " 10 "
Rhamnose*....	5.0	5.19	5.30	4.72	4.82	94.4	96.4	0.1 " 10 "
Arabinose.....	5.0	6.22	6.30	4.67	4.73	93.4	94.6	0.05 " 5 "
Lyxose*.....	5.0	6.52	6.45	4.89	4.84	97.8	96.8	0.05 " 5 "
Ribose*.....	5.0	6.57	6.56	4.93	4.92	98.6	98.4	0.05 " 5 "
Xylose.....	5.0	6.47	6.53	4.85	4.90	97.0	98.0	0.1 " 10 "
Cellobiose.....	5.0	2.83	2.93	4.84	5.02	96.8	100.4	0.05 " 10 "
Lactose.....	5.0	2.82	2.99	4.83	5.12	96.6	102.6	0.05 " 5 "
Maltose.....	5.0	2.59	2.78	4.43	4.76	88.6	93.2	0.05 " 5 "

\* Procedure B (see text) was used for these sugars and Procedure A for the others.

glass-stoppered 4 dm. tubes and readings were made at intervals without removing the samples from the tubes. For the longer intervals, new samples were taken from the glass-stoppered volumetric flasks containing the original mixture which was kept at the same temperature as the tubes. The optical data are plotted in Fig. 2.

In order to make certain that the initial readings were independent of the form of the sugar used (whether  $\alpha$  or  $\beta$ ) the rate of

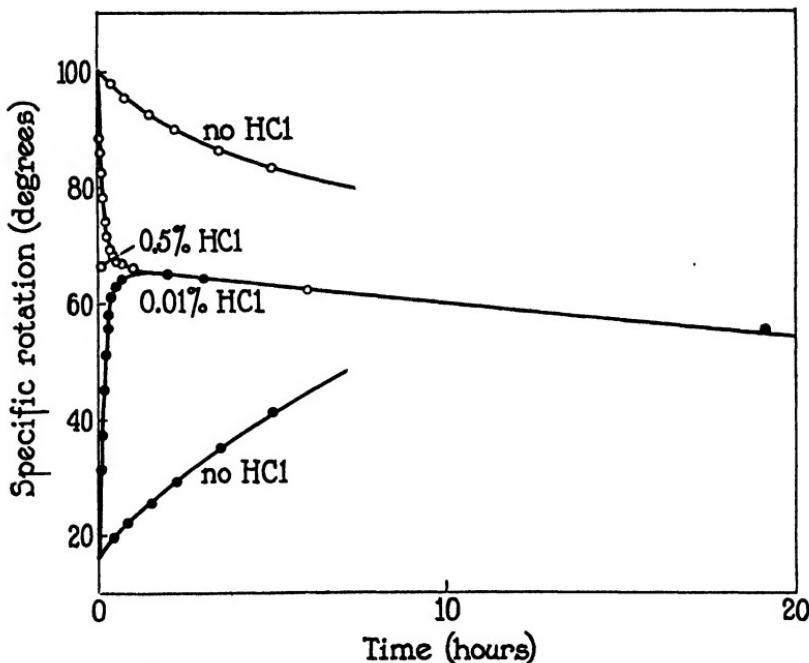


FIG. 3. Mutarotation of  $\alpha$ - and  $\beta$ -glucose at 25° in methyl alcohol

mutarotation of the two forms of glucose in methyl alcohol was determined and the effect of varying amounts of hydrogen chloride was studied. It may be seen from Fig. 3 that the mutarotation is so rapid in the presence of even 0.01 per cent hydrogen chloride that in the presence of 0.5 per cent, the mutarotation would be almost instantaneous and the first reading would represent the equilibrium mixture of the free sugar independently of whether the  $\alpha$  or  $\beta$  form is employed.

**SUMMARY**

The glucoside formation of all the commoner monoses has been studied under comparable conditions.

For each sugar the proportion of furanoside rises to a maximum and then decreases. The time required to reach this maximum is in general different for each individual sugar.

The specific rates of furanoside and pyranoside formation are different for each sugar. The ratio of the two rates is likewise in general different for each sugar.



## THE RECOVERY PROCESS AFTER EXERCISE IN THE MAMMAL

### II. THE CONVERSION OF INFUSED *d*-LACTIC ACID INTO MUSCLE GLYCOGEN

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#### INTRODUCTION

The formation of glycogen from *d*-lactates added to frog muscle pulp was demonstrated by Meyerhof, Lohmann, and Meier (1). Associated with this was an increased oxygen consumption. The same authors also state that perfusion of the hind limbs of the frog with lactic acid solutions of pH 7.4 results in muscle glycogen synthesis. Eggleton and Evans (2) have recently repeated these perfusion experiments with negative results.

In the mammal, attempts to demonstrate muscle glycogen synthesis following injection of lactic acid have been even less successful. Janssen and Jost (3) infused racemic lactates into the jugular vein of unanesthetized dogs in which a transection of the lower portion of the spinal cord had been previously performed. They found that although lactic acid rapidly disappeared from the blood there was no increase in oxygen consumption and no formation of muscle glycogen. Previous to their work Elias and Schubert (4) had shown that the intraarterial injection of lactic acid into the hind limbs of dogs did not result in any synthesis of glycogen.

Abramson, Eggleton, and Eggleton (5) and Eggleton and Evans (2) report that they were unable to detect muscle glycogen formation after the injection of lactates into dogs.

\* Working with the aid of a grant from the Banting Research Foundation.

There are two obvious possibilities for the negative results obtained in mammals: (1) Many workers have used racemic lactic acid. It is now well known that only the dextrorotatory lactic acid is used by the mammal (Meyerhof, Lohmann, and Meier (1); Cori and Cori (6)). (2) The mammalian liver can convert *d*-lactic acid into glycogen with great facility. Mandel and Lusk (7) in 1906 showed that the phlorhizinized dog can at times quantitatively convert *d*-lactic acid into glucose and more recently Cori and Cori (6) have shown that *d*-lactic acid fed or injected into rats is to a great extent converted into liver glycogen.

In the work reported here the decapitated or spinal eviscerated cat was used. In these preparations the liver although left *in situ* is not functioning. The preparations from the point of view of this work consist chiefly of the muscles perfused with their own blood. The lactic acid infused was the dextrorotatory variety and was very kindly prepared for us by Professor W. H. Peterson of the University of Wisconsin. With the use of this preparation under certain conditions well marked muscle glycogen synthesis can be demonstrated when *d*-lactic acid is infused.

#### *Methods*

*Animals*—The animals used in all experiments were cats. These were either decapitated by Sherrington's technique or all the vessels supplying the head were ligatured in the manner described by Bodo and Marks (8). This was done under ether anesthesia. After preparation by one of the above methods the animals were eviscerated, the kidneys removed, and the adrenal veins ligatured. In some cases the adrenal glands were removed.

After decapitation artificial respiration was maintained by a Palmer Ideal pump. When the evisceration was completed the animal was switched onto a Schuster circulating respirometer. The ventilation was kept at between 300 and 400 cc. a minute for a cat of original weight of 3 kilos and was altered to suit the varying weights of the animals.

*Removal of Muscles*—As we wished to calculate the total glycogen formation in our preparations, it was necessary to analyze more than one muscle from each side. In our earlier experiments we removed four muscles from each side. These were the gastrocnemius, soleus, sartorius, and the rectus femoris.

In the majority of our experiments we wished to estimate not only glycogen but also lactic acid and glucose in the muscles. In order to cut down the number of analyses and also to avoid a certain amount of trauma, unavoidable in the first method, we removed, after suitable preparation, all the muscles of the calf. The manner in which this was done was as follows:

The skin over the calf was split longitudinally and freed from the underlying muscles. The fascia over the gastrocnemius was then split so that the hamstrings might be cut at their insertions. These manipulations were carefully and rapidly carried out and the skin closed until it was desired to remove the muscles. At this time the skin was cut across at the ankle joint and the whole calf was suddenly immersed in a slush of solid carbon dioxide and ethyl chloride or ether. This method was introduced by Davenport and Davenport (9) and resulted in almost instantaneous freezing of the calf muscles. The slush was left around the calf until it was solid. A stout ligature was then tied tightly around the knee so as to occlude the arteries and this joint was disarticulated with a heavy knife, after first sawing through the calf just proximal to the ankle joint.

The frozen calf was then split longitudinally and immediately placed in a large amount of CO<sub>2</sub> snow. The muscles were then split off from the tibia and fibula and replaced in the CO<sub>2</sub> snow. These large muscle pieces were then cut into small bits one by one and these fragments were divided equally among three tared beakers which were immersed in CO<sub>2</sub> snow.

The outside of the beakers was then scraped clear of ice and snow and reweighed. The appropriate reagents were then added to each specimen.

In our hands this method has given satisfactory results. Not only is the amount of trauma much lessened but analysis of all the calf muscles must give a very representative value for the whole musculature of the body. Actually about fourteen different muscles are analyzed instead of the usual four or five. Determinations of the same constituent in all three samples gave very consistent results. Thus, in one control experiment, the glycogen content of the three final specimens from one limb was 0.866, 0.878, and 0.857 gm. per cent, respectively. Analyses for other constituents gave similar results.

*Liver Samples*—When liver samples were required the first specimen was removed between bamboo strips, divided into three portions, weighed, and chopped up at once in the appropriate solutions. The second specimen was obtained from the remainder of the same lobe.

*Chemical Methods*—Muscle glycogen was determined on one specimen of calf muscles by Pflüger's method. The boiling 60 per cent KOH was poured over the frozen muscle fragments and the whole mixture rapidly heated to boiling. After hydrolysis the solution was poured into 250 cc. Pyrex centrifuge tubes and diluted to 70 cc. with water. 140 cc. of 94 per cent alcohol were added and the mixture allowed to stand overnight. The next day the tubes were centrifuged and the supernatant fluid poured off. After neutralization, addition of 50 cc. of 2.5 per cent HCl, and hydrolysis in a water bath, the tubes were cooled and neutralized. The contents were then diluted to suitable volume in volumetric flasks, filtered, and the glucose estimated by the Shaffer-Hartmann macro method. Glycogen is expressed as glucose throughout.

Muscle lactic acid was determined in the second specimen of the calf muscles. Ice-cold 5 per cent trichloroacetic acid was poured over the fragments and they were chopped into a mash with scissors. The mixture was then allowed to freeze solid by immersion of the beaker in CO<sub>2</sub> snow. After thawing at room temperature the mixture was diluted to 100 cc. with more trichloroacetic acid and allowed to stand in the ice box overnight. It was assumed that by this time the lactic acid was equally distributed between the muscle pulp and the fluid. The mixture was then filtered, an aliquot portion neutralized, treated with CuSO<sub>4</sub> and Ca(OH)<sub>2</sub>, and the lactic acid estimated by the method of Friedemann, Cotonio, and Shaffer.

Muscle glucose was determined on the third specimen by the method of West, Scharles, and Peterson (10) with such modifications as were necessary when dealing with tissues instead of blood. The preliminary procedures were the same as those for muscle lactic acid except that 2 per cent H<sub>2</sub>SO<sub>4</sub> was used as a fixative instead of trichloroacetic acid. After freezing and thawing the mixture was made up to 100 cc. and stood in the ice box overnight. The next day the mixtures were filtered, and to about 60 cc. of the filtrate 10 cc. of the West, Scharles, and Peterson reagent were added. The mixture was then neutralized with BaCO<sub>3</sub> and

filtered. After treatment with zinc dust and  $\text{Na}_2\text{SO}_4$ , and refiltering the glucose was estimated by Somogyi's modification of the Shaffer-Hartmann method.

Analysis of the liver specimens for glycogen, lactic acid, and glucose was carried out in the same manner.

Blood analyses were made on blood withdrawn from a carotid artery. Glucose and lactic acid were determined in the filtrates obtained by the use of Folin's unlaked blood method, glucose by means of Somogyi's method, and lactic acid by the method of Friedemann, Cotonio, and Shaffer. In some experiments plasma fats were estimated by the method of Stewart, Gaddie, and Dunlop (11).

*Procedure of Experiments*—In all the experiments reported here the following technique was adhered to. After completion of the preparation respiration was carried on for some time in order to remove all traces of ether. During this time the tared beakers for the various muscle and liver samples were cooled.

The preparation was then switched onto the Schuster respirometer and the rate and ventilation suitably adjusted. One or two 15 minute periods of oxygen utilization were then recorded. During this time the perfusion pump was filled with the solution to be infused.

The liver sample was then removed. This was followed by the removal of the previously prepared calf and lastly a blood sample was withdrawn from the carotid artery.

Immediately after the withdrawal of the blood sample the perfusion pump was started and simultaneously the recorder on the respirometer was set at zero so that all oxygen utilization from this point might be calculated. Readings of oxygen consumption, room temperature, and barometric pressure, as well as the cat's blood pressure and temperature, were made at 15 minute intervals during the entire period of infusion.

After a sufficient time, usually between 3 to 4 hours, the second samples were taken. The order was changed here, the blood being taken first, then the muscle and lastly the liver.

### Results

Five groups of experiments have been performed: (1) A group in which glucose alone was infused. (2) A group in which glucose and insulin were infused. These two groups were done for

control purposes only as any deductions to be drawn from them have already been adequately dealt with by Best, Dale, *et al.* (12). (3) A group in which *d*-lactic acid was infused. (4) A group in which *d*-lactic acid and glucose were infused. (5) A group in which *d*-lactic acid, glucose, and insulin were infused.

In the majority of the experiments in these various groups the changes in muscle glycogen, glucose, and lactic acid were followed. The variations in blood sugar and lactic acid were also estimated. In addition the oxygen consumption was measured and in some cases the carbon dioxide production. With these facts before us it is possible (if certain assumptions are made) to draw up a balance sheet for the glucose and lactic acid in the manner done by Best, Dale, *et al.* (12) and by Corkill and Marks (13). The question of these balance sheets will be discussed later. First let us consider such findings as are not the subject of controversy at the present time (*cf.* Kilborn (14); Corkill, Dale, and Marks (15)).

#### *Muscle Glycogen Changes*

*No Infusion, Glucose, or Glucose and Insulin Infused*—These experiments are tabulated in Table I. They show that if nothing is infused, or if glucose alone is infused, the muscle glycogen remains unchanged over a period of several hours.

If glucose and insulin are infused then, as has been previously shown (12), there is a well marked increase in muscle glycogen.

*d-Lactic Acid Infused*—Fourteen experiments have been performed in which the changes in muscle glycogen have been followed during the infusion of *d*-lactic acid. The lactic acid has been used in various strengths, from 2 to 10 per cent and in most experiments has been half or two-thirds neutralized with NaOH before use. In three experiments a 5 per cent solution of the free acid was used. The rate of infusion varied from a minimum value of 13 cc. an hour to a maximum one of 27 cc. an hour. This maximum rate was only used in a few experiments. In the majority between 12 and 13 cc. an hour were infused. No untoward effects were observed as a result of the infusion, the blood pressure and general condition of the preparations remaining quite satisfactory throughout the infusion.

A study of Table II shows that in five experiments the increase in muscle glycogen was between 0.05 and 0.10 gm. per cent. In

another four experiments the increase was less than 0.05 gm. per cent and in the remainder either no change was observed or else slight falls in muscle glycogen were measured. It is extremely

TABLE I  
*Effect of No Infusion, Infusion of Glucose, and Infusion of Glucose and Insulin upon Muscle Glycogen in Decapitated Eviscerated Cat*

Cat No.	Weight kg.	Glucose infused gm.	Time of infusion hrs.	Muscle glycogen		Muscles analyzed
				Before gm. per 100 gm.	After gm. per 100 gm.	
<b>No infusion</b>						
46 O.S.*	2.75	Nil		0.760	0.730	4 muscles
47 "	3.25	"		0.845	0.781	4 "
48 "	2.93	"		0.671	0.657	4 "
54 N.S.*	2.53	"		0.858	0.813	Whole calf
Average.....				0.783	0.745	
<b>Glucose infused</b>						
16 N.S.	2.75	2.46	3½	0.626	0.644	Whole calf
32 "	3.20	1.72	2½	0.881	0.855	" "
33 "	3.40	2.31	3½	0.564	0.576	" "
52 "	2.91	2.06	3½	0.620	0.645	" "
53 "	2.70	2.19	3½	0.948	0.867	" "
Average.....				0.728	0.717	
<b>Glucose and insulin infused</b>						
19 N.S.	3.30	3.85	3	0.584	0.715	Whole calf
26 "	2.80	5.15	4	0.685	0.840	" "
34 "	3.00	5.36	4	0.497	0.725	" "
54 O.S.	2.25	3.84	2	0.617	0.834	4 muscles
Average.....				0.596	0.778	

\* N. S. and O. S. refer to the serial numbers of the cats.

doubtful if changes in muscle glycogen of the order of 0.05 gm. per cent can be accurately measured by the method employed.

It is to be concluded, therefore, that the infusion of *d*-lactic

acid alone into decapitated eviscerated cats causes in the majority of experiments no increase in muscle glycogen.

*Glucose and d-Lactic Acid Infused*—It occurred to us that the reason for the small or absent muscle glycogen synthesis following the infusion of *d*-lactic acid alone might be due to the fact that, owing to the deficient glucose stores of the decapitated eviscerated

TABLE II  
*Effect of Infusion of d-Lactic Acid upon Muscle Glycogen in Decapitated Eviscerated Cat*

Cat No.	Weight	<i>d</i> -Lactic acid infused	Time of infusion	Muscle glycogen		Notes
				Before gm. per 100 gm.	After gm. per 100 gm.	
17 N.S.	3.10	3.40	3½	0.623	0.565	10 per cent solution, $\frac{1}{2}$ neutralized
18 "	2.20	3.70	2½	0.513	0.470	10 " " " $\frac{1}{2}$ "
28 "	2.95	4.78	3½	0.774	0.684	10 " " " $\frac{1}{2}$ "
60 O.S.	3.00	6.19	3	0.671	0.672	7.5 " " " $\frac{1}{2}$ "
59 "	3.58	5.44	2½	0.537	0.599	7.5 " " " $\frac{1}{2}$ "
58 "	2.70	3.22	2½	0.582	0.614	7.5 " " " $\frac{1}{2}$ "
53 "	2.65	3.29	2	0.419	0.471	5 " " " $\frac{1}{2}$ "
55 "	2.25	3.70	3	0.702	0.741	5 " " " $\frac{1}{2}$ "
46 N.S.	3.10	1.88	2½	0.597	0.672	5 " " " $\frac{1}{2}$ "
47 "	2.71	2.37	3½	0.796	0.882	5 " " " $\frac{1}{2}$ "
51 O.S.	2.60	3.95	3	0.421	0.467	5 " " " of free acid
38 N.S.	3.15	2.77	4½	0.696	0.636	5 " " " " " "
41 "	2.40	1.33	2	0.578	0.676	5 " " " " " "
20 "	2.85	1.03	3½	0.564	0.480	2 " " " " $\frac{1}{2}$ neutralized
<b>Average.....</b>				<b>0.605</b>	<b>0.616</b>	

preparation, the lactic acid was being oxidized to supply the metabolic requirements. Therefore, in a further series of six cats, we perfused equal amounts of glucose along with the lactic acid and studied the effect upon the muscle glycogen. The first three experiments (Cats 35, 36, and 44 N.S., Table III) seemed to indicate that under these conditions well marked muscle glycogen deposition occurred. Three more similar experiments were then performed (Cats 45, 48, and 49 N.S., Table III) but in none of

TABLE III

*Effect of Glucose and d-Lactic Acid and Glucose, d-Lactic Acid, and Insulin upon Muscle Glycogen in Decapitated Eviscerated Cat*

Cat No.	Weight kg.	Glucose infused gm.	d-Lactic acid infused gm.	Time of infusion hrs.	Muscle glycogen		Notes
					Before gm. per 100 gm.	After gm. per 100 gm.	
<b>Glucose and d-lactic acid</b>							
35 N.S.	2.75	2.11	2.11	3½	0.706	0.866	5 per cent glucose, 5 per cent free lactic acid infused
36 "	3.50	2.61	2.61	4	0.808	0.905	" "
44 "	2.70	2.04	2.04	3	0.842	0.985	5 per cent glucose, 5 per cent $\frac{1}{2}$ neutralized lactic acid infused
45 "	2.80	2.06	2.06	3	0.675	0.683	" "
48 "	3.08	2.28	2.28	3½	0.596	0.495	" "
49 "	2.55	1.76	1.76	2½	0.393	0.373	" "
Average.....					0.670	0.718	
<b>Glucose, d-lactic acid, and insulin</b>							
37 N.S.	3.40	2.79	2.79	4½	0.895	1.201	5 per cent glucose, 5 per cent neutralized lactic acid infused
42 "	3.35	5.18	2.59	4	0.637	1.000	10 per cent glucose, 5 percent $\frac{1}{2}$ neutralized lactic acid infused
43 "	2.75	3.46	1.73	2½	0.621	0.838	" "
50 "	2.91	4.13	2.06	3	0.571	0.864	" "
51 "	2.88	4.36	2.18	3½	0.764	1.022	" "
Average.....					0.698	0.985	

there were any significant amounts of glycogen deposited. We are at a loss to account for the difference in these two groups since the same technique was adhered to in each. Nevertheless, the first three experiments do show that synthesis of glycogen can occur when glucose and *d*-lactic acid are infused together into the decapitated eviscerated cat. Such well marked synthesis has never been observed when glucose alone is infused, so presumably the substance responsible in these three positive experiments was the *d*-lactic acid.

*Glucose, d-Lactic Acid, and Insulin Infused*—The well marked muscle glycogen synthesis obtained in some experiments in the above group encouraged us to try the effect of infusion of insulin along with the glucose and *d*-lactic acid. Most observers (12, 16) are agreed that the infusion of glucose and insulin into a decapitated, eviscerated preparation leads to the use of carbohydrate only to supply the metabolic requirements. Therefore, if a carbohydrate plethora is favorable to the conversion of *d*-lactic acid into muscle glycogen, it should readily occur in these experiments in which the *d*-lactic acid is infused along with glucose and insulin. Five experiments of this kind have been performed (Table III). In all of them a large deposition of muscle glycogen occurred. Comparison of the results with the increases in muscle glycogen after glucose and insulin only (Table I) shows that the formation of muscle glycogen in the lactic acid experiments is much greater than would be expected from the amounts of glucose infused.

This difference is more clearly demonstrated in Table IV. In this table the actual glucose and lactic acid utilization as calculated from the various analyses of blood and muscle is shown.<sup>1</sup> In addition the total glycogen formation is calculated. Now in all these experiments sufficient glucose was supplied to keep the blood sugar at or above the initial level. It is therefore presumed that the glucose utilization was at a maximum in each preparation. In the last column of Table IV is shown the total glycogen formation expressed as a percentage of the total glucose utilization.

It will be seen that under the experimental conditions used in

<sup>1</sup> In making these calculations the blood volume was taken as 6.2 per cent of the original body weight and the total muscle weight as 50 per cent of the original body weight. These were the figures used by Best, Dale, et al. (12).

the glucose and insulin experiments an average of 50 per cent of the glucose utilized could be accounted for as muscle glycogen. Best, Dale, Hoet, and Marks' (12) results show a similar percentage glycogen formation in those experiments which are most comparable to ours. Thus, in Experiment 5 in their paper, the percentage glycogen formation was 52.5 per cent, and in their Experiment 6 it was 47 per cent.

However, when we examine these experiments in which *d*-lactic acid was given, it will be seen at once that in three out of five ex-

TABLE IV

*Percentage Glycogen Formation from Glucose and Insulin, and from Glucose, d-Lactic Acid, and Insulin*

	Cat No.	Glucose utilization gm.	Lactic acid utilization gm.	Total glycogen formation gm.	Glycogen formed × 100 Glucose utilization
					per cent
Glucose and insulin	19 N.S.	4.24	0.11	2.16	51
	26 "	4.60	0.09	1.84	40
	34 "	5.70	0.45	3.42	60
	Mean.....				50
Glucose, <i>d</i> -lactic acid, and insulin	37 N.S.	4.05	2.81	5.20	129
	42 "	5.89	2.13	6.08	104
	43 "	3.27	1.73	2.98	91
	50 "	5.19	1.50	4.21	81
	51 "	5.39	1.86	3.72	69
	Mean.....				95

periments the glycogen formation is either greater than or equal to the total glucose utilized. In the other two experiments the percentage glycogen formation is much greater than it is in similar experiments with glucose and insulin only.

The only possible conclusion is that it is under these conditions when the muscles are probably utilizing carbohydrate only that the conversion of *d*-lactic acid to muscle glycogen readily occurs. As will be shown later, insulin has no effect on the amount of lactic acid utilized, and other experiments have convinced us that it does not cause any appreciable muscle glycogen synthesis in animals infused with *d*-lactic acid only.

*Utilization of d-Lactic Acid in Decapitated Eviscerated Cat*

In spite of the fact that they were unable to detect muscle glycogen synthesis following lactic acid infusion, all workers (3, 5) are agreed that there is a marked utilization of infused lactic acid in the mammal. This was determined by its disappearance from the blood (Janssen and Jost (3)) and also by the increase in the CO<sub>2</sub>-combining power of the plasma when sodium lactate was infused (Abramson, Eggleton, and Eggleton (5)).

In some earlier experiments one of us (17) infused racemic lactates into mammals and came to the conclusion that removal of lactate was greatly decreased or abolished after evisceration. We now realize that at that time one important source of error was overlooked. This was the effect of artificial ventilation on the blood lactic acid in eviscerated preparations. It has since been abundantly demonstrated that overventilation in such preparations causes a marked accumulation of lactic acid in the blood (Kilborn, Soskin, and Thomas (18); Eggleton and Evans (19)). This factor was not properly controlled in these earlier experiments and we must therefore now regard these previous conclusions as erroneous.

In the present experiments where the ventilation was carefully controlled in order to avoid this error, and where the muscles, blood, and in some cases the skin were analyzed for lactic acid, there is no doubt about the utilization of *d*-lactic acid in the decapitated eviscerated cat.

In Table V are grouped together the figures for the utilization not only of *d*-lactic acid but also of glucose in the various groups of experiments performed. It will be seen that in all experiments there was a utilization of *d*-lactic acid at the rate of about 0.20 gm. per kilo of original body weight per hour. It will be further observed that the simultaneous infusion of glucose or glucose and insulin along with the *d*-lactic acid is without effect upon the amounts of lactic acid utilized. The average utilization rate of glucose alone is about the same as that of *d*-lactic acid alone; *i.e.*, 0.20 gm. per kilo per hour. The addition of insulin doubles and in some cases nearly trebles the utilization of glucose. All these utilization rates for *d*-lactic acid and glucose are maximum ones, since the blood glucose and lactic acid were either constant or increasing during the period of infusion.

We may now ask, what is the fate of the *d*-lactic acid that disappears in these preparations? First of all, in those experiments in which it is most reasonable to assume a R.Q. of unity, i.e. those

TABLE V  
*Utilization of Glucose and d-Lactic Acid with and without Insulin*

Cat No.	Total glucose utilization	Glucose utilization	Total <i>d</i> -lactic acid utilization	<i>d</i> -Lactic acid utilization	Cat No.	Total glucose utilization	Glucose utilization	Total <i>d</i> -lactic acid utilization	<i>d</i> -Lactic acid utilization
Glucose infused					<i>d</i> -Lactic acid infused				
16 N.S.	2.42	0.23			17 N.S.			1.96	0.19
32 "	1.86	0.23			18 "			2.56	0.42
33 "	2.27	0.19			20 "			1.30	0.12
52 "	2.49	0.27			28 "			2.50	0.24
Mean.....	0.23				38 "			3.00	0.22
Glucose and insulin infused					41 "			1.41	0.29
19 N.S.	4.24	0.43			46 "			1.51	0.24
26 "	4.60	0.41			47 "			2.02	0.21
34 "	5.70	0.48			Mean.....				0.24
Mean.....	0.44				Glucose and lactic acid				
Glucose, <i>d</i> -lactic acid, and insulin infused					35 N.S.	2.40	0.27	1.94	0.22
42 N.S.	5.89	0.44	2.13	0.16	36 "	2.88	0.21	2.43	0.17
43 "	3.27	0.48	1.73	0.25	44 "	1.67	0.20	1.92	0.23
50 "	5.19	0.55	1.50	0.16	45 "	1.84	0.22	1.92	0.23
51 "	5.39	0.57	1.86	0.20	48 "	1.97	0.20	1.07	0.11
Mean.....	0.51				49 "	0.80	0.11	1.32	0.19
Mean.....	0.51				Mean.....	0.20			0.19

\* This signifies gm. per kilo of original body weight per hour of infusion.

in which glucose, lactic acid, and insulin are given, the greater part of the glucose and lactic acid that is utilized can be accounted for in terms of oxidation or glycogen deposition. The following

experiment exemplifies this. Into a cat weighing 2.75 kilos, a total of 3.455 gm. of glucose and 1.728 gm. of *d*-lactic acid (5 per cent solution, half neutralized with NaOH) was infused over a period of 2½ hours. In addition, 20 units of insulin were injected intravenously. The blood sugar rose from 0.176 to 0.303 gm. per cent and the lactic acid from 0.011 to 0.038 gm. per cent. The

	Before infusion	End of infusion
	gm. per cent	gm. per cent
Muscle glycogen.....	0.621	0.838
"    glucose.....	0.064	0.078
"    lactic acid.....	0.102	0.099
Liver glycogen.....	0.916	0.967
"    glucose.....	2.435	2.145

Liver weight, 90.2 gm.

*Balance Sheet*

	gm.		gm.
Glucose infused.....	3.46	Glucose equivalent of oxygen used.....	2.06
<i>d</i> -Lactic acid infused.....	1.73	Rise in blood glucose, 0.127 × 1.71 .....	0.22
Fall in muscle lactic acid, 0.004 × 13.8.....	0.06	Rise in blood lactic acid, 0.027 × 1.71.....	0.05
Glucose from liver, 0.239 × 0.90.....	0.22	Rise in muscle glucose, 0.014 × 13.8.....	0.19
Total glucose and lactic acid disappearing.....	5.47	Increase in muscle glycogen, 0.217 × 13.8.....	3.00
		Total glucose and lactic acid accounted for.....	5.52

total oxygen consumption was 1560 cc. at standard temperature and pressure corresponding to the combustion of 2.06 gm. of glucose if a R.Q. of unity is assumed.

Secondly, in those experiments in which *d*-lactic acid was infused alone or with glucose, the utilization was of the same order as in experiments with glucose and insulin (Table V), yet in these experiments the disappearance of lactic acid cannot be accounted for by glycogen formation (Tables II and III). It may have been

oxidized or converted into other substances which the present analyses did not include. The respiratory quotient is obviously of little value in experiments in which both acid and base have been infused and, furthermore, the assumption of a R.Q. of unity in these experiments is still the subject of some controversy, even when glucose only is supplied (14, 15). We have therefore hesitated at the present time to draw any conclusions from balance sheets constructed for experiments in which lactic acid alone or with glucose was infused.

Lusk (20) has raised the question as to whether lactic acid *per se* is oxidizable. If it is not, then in the decapitated eviscerated cat the amounts of *d*-lactic acid utilized and not accountable for as muscle glycogen may have been transformed into intermediary substances which, if ample carbohydrate is available, are then further synthesized into glycogen. So far we have not studied these possible intermediaries since in many of our experiments in which lactic acid alone is infused the assumption of a R.Q. of unity gives a fairly satisfactory balance sheet, and other types of experiments are desirable to settle this point.

It will be recalled that Janssen and Jost (3) failed to find any increase in oxygen consumption of intact dog muscles during lactate infusion, and Abramson, Eggleton, and Eggleton (5) found only small increases in the total oxygen consumption of dogs under amytal. In our experiments similar findings were observed. In only two cases was the increase in oxygen consumption greater than could be accounted for by the improvement in the circulation that occurs when lactate solutions are infused, particularly when the infusion is begun after withdrawal of blood.

#### *Minor Observations*

It was observed in the majority of experiments when lactate was infused that the concentration in the blood at the end of the experiment was greater than that in the muscles. Some-what similar findings are reported by Eggleton and Evans (2) on blood and muscle lactic acid after exercise in dogs.

In a few experiments samples of the skin were removed at the beginning and end of the perfusion and analyzed for lactic acid. In one such experiment where 5 per cent *d*-lactic acid and 5 per cent glucose were infused the concentration in the blood and skin

at the beginning of the experiment was 0.031 gm. per cent of lactic acid in each. At the end the concentration was 0.056 gm. per cent both in skin and blood. Evidently there is not an excessive accumulation of this substance in the skin when it is given at a slow constant rate.

Muscle glucose analyzed by the method outlined above was always much lower than blood glucose; usually it was only 25 to 30 per cent of the values found in the blood. Similar results were obtained by Trimble and Carey (21) in intact animals.

In many of the experiments plasma fats were determined by the modified method of Stewart, Gaddie, and Dunlop (11). In a few experiments well marked falls were observed but in the majority the changes were only slight. It might be added that the two largest decreases, 0.103 and 0.215 gm. per cent, respectively, were observed in decapitated eviscerated preparations which were not infused but left untouched for 4 hours.

Although we analyzed the liver for lactic acid both before and after the infusion in all our lactic acid experiments, we never found more than insignificant amounts had diffused back into it from the blood. The lactic acid content always rises in the livers of eviscerated animals, due no doubt to glycolysis.

#### DISCUSSION

Meyerhof (22) has recently stated that in the chemical processes involved in the resynthesis of muscle glycogen the oxidation of lactic acid was not requisite for the resynthesis of the remaining lactic acid; instead carbohydrate may be burnt. Lusk (20), on the other hand, maintains that the oxidation of fat may also supply the necessary energy, particularly when carbohydrate is lacking.

The experiments reported here would indicate that an almost totally carbohydrate type of muscle metabolism is the most favorable for lactic acid synthesis to muscle glycogen. This is shown by the constant synthesis of glycogen from lactic acid in the decapitated eviscerated cat when glucose and insulin are also supplied. With glucose alone synthesis can still be observed but it is not so striking or constant a finding as it is in the glucose and insulin experiments. On the other hand, *d*-lactic acid alone seems to have but little power to form muscle glycogen in preparations in which the carbohydrate stores are much depleted, although it

can be assumed that ample fat and protein are available. In addition the changes in plasma fat are but slight compared to the rapid fall in blood sugar that occurs in these preparations.

It might be objected that there may be a difference between resting muscles perfused with blood rich in added lactates, and exercising muscles in which the lactic acid is produced in intimate relation with the cells. Nevertheless it has been amply demonstrated that large amounts of lactic acid may leave the exercising limbs in the mammal and be carried by the blood to resting muscles. These resting muscles must then be comparable to those in our preparations, as regards their ability to deal with this lactic acid.

Long and Grant (23) have shown in the 24 hour-fasted rat where carbohydrate stores are low, that after exercise although the lactic acid is rapidly removed the resynthesis of muscle glycogen is extremely slow. More recent unpublished experiments indicate that the feeding of glucose after exercise rapidly restores the muscle glycogen while *d*-lactic acid only slightly accelerates its rate of restoration.

Debois (24) has recently investigated the restoration of muscle glycogen after exercise in the decapitated cat. As had been previously demonstrated by McKay (25), there is practically no resynthesis after several hours in such a preparation. Debois confirms this finding, and in addition shows that in the intact cat under ether, section of the vagi also prevented this resynthesis although recovery occurred with intact vagi. In addition, pancreatectomy prevented the resynthesis although this could again be observed if insulin was injected. He concludes that the recovery process in mammalian muscle is a insulin function. Section of the vagi or decapitation abolishes this since the secretion of insulin is under the control of the vagus nerves.

These experiments have an obvious parallel with ours, since so far as we know the action of insulin is primarily upon carbohydrates, and it is when our preparations were chiefly utilizing these substances that the most marked synthesis of muscle glycogen from *d*-lactic acid was observed. The recovery process in muscle after exercise is not confined solely to the restitution of glycogen and the removal of lactic acid. Nevertheless, if in the intact mammal the resynthesis of muscle glycogen after exercise requires

the participation of glucose and insulin, then this phase of recovery is not entirely dependent upon a cycle of chemical changes confined to the muscles alone. The liver participates by supplying glucose, as is clearly shown by some recent results obtained by Miss R. Grant in this laboratory. She has shown that after exercise in rats with a high initial liver glycogen content there is a steady decrease in liver glycogen, not only during exercise but also throughout a recovery period of 5 hours. The liver may also convert lactic acid escaping from the muscles into glucose. The utilization of glucose from either of these sources further requires an ample supply of insulin and presumably would be impaired in a diabetic animal (*cf.* Debois (24)) These observations do not exclude the possibility that the *exercising* muscles themselves may also convert lactic acid formed within them into glycogen without the intervention of other organs, even though the bulk of the evidence is against *resting* muscles being able to bring about this synthesis from lactic acid brought to them by the blood.

It is our belief that the processes of recovery from exercise are a function of the organism as a whole and are not entirely determined by the muscles themselves.

We wish to acknowledge the assistance afforded us by Mrs. E. M. Venning in certain of the analyses.

#### SUMMARY

1. Infusion of *d*-lactic acid alone into decapitated eviscerated cats either does not cause muscle glycogen deposition or causes it only in insignificant amounts.
2. Infusion of glucose and *d*-lactic acid together leads in some experiments to well marked muscle glycogen deposition.
3. Infusion of glucose, *d*-lactic acid, and insulin together causes a much greater muscle glycogen deposition than is to be expected from glucose and insulin in equivalent amounts.
4. The rate of utilization of *d*-lactic acid in the decapitated eviscerated cat is about 0.2 gm. per kilo of original body weight per hour. This is not altered by the addition of glucose or insulin.
5. Reasons are given for the belief (*a*) that the synthesis of muscle glycogen from *d*-lactic acid is favored by an exclusively carbohydrate type of muscle metabolism, and (*b*) that the re-

covery process in the intact mammal is a function of the organism as a whole rather than one affected by the muscles only.

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## THE DETERMINATION OF SUGARS IN PLANT EXTRACTS\*

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In order to be able to make a satisfactory choice among the methods available for the determination of sugars in plant extracts and to make the best possible interpretation of the results obtained, it seemed necessary to study the following points. (1) The comparative action of a number of oxidizing reagents on a variety of plant materials. (2) The behavior of these reagents toward sucrose hydrolyzed by invertase. (3) The most reliable method for the calculation of sucrose hydrolyzed by invertase when determined in the presence of reducing sugars.

In this paper are reported the results obtained by the use of four oxidizing solutions, including three differing degrees of alkalinity.

*Fehling's Solution*—The modification recommended by Quisumbing and Thomas (1) was used, and their procedure for reduction was followed except that 250 cc. beakers were used. The reduced copper was determined by the Bertrand titration method. Sucrose was hydrolyzed in a volume of 50 cc. in the beaker in which the determination was to be made. The solution was made just acid to methyl red with acetic acid, and 3 drops of a 1 per cent solution of Wallerstein's Red Label invertase scales were added. Under these conditions at least 25 mg. of sucrose are hydrolyzed in 2 hours at room temperature.

*Shaffer-Hartmann Micro Reagent* (2)—This solution was used in 10 cc. portions added to 10 cc. of the sugar solution. The tubes were covered with inverted 100 cc. beakers and heated in the boiling water bath for 20 minutes.

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*Tompsett's Reagent* (3)—In a study of the Scales method Sullivan (4) found that iodine liberated in the presence of plant extracts may be absorbed by some constituent of the extract, such as phlorhizin, causing an error in the determination of the reduced copper. The Tompsett modification of the Shaffer-Hartmann solution would avoid this difficulty since the cuprous oxide is separated before its solution in the acidified iodate solution. Due to autoreduction in all samples of Tompsett's solution a few days after preparation, it was necessary to separate the CuSO<sub>4</sub> from the other constituents, making each part double the original concentration; 5 cc. of each were used for a determination.

*Bicarbonate Solution*—In order to determine the effect of a still less strongly alkaline oxidizing solution, a bicarbonate reagent was prepared. The final ratio of carbonate to bicarbonate was that found by Somogyi (5) to give the lowest ratio of sugar to reduced copper. The concentration of tartrate was increased, as suggested by Tompsett. Because of autoreduction it was necessary to make this reagent in two parts as follows:

	gm. per l.
Part A. Cupric sulfate crystals.....	20.0
"    B. Tartaric acid.....	30.0
"    Sodium carbonate (anhydrous).....	61.2
"    bicarbonate.....	50.0

The carbonate and tartaric acid were dissolved together in hot water in a volumetric flask. The solution was boiled gently to expel CO<sub>2</sub>. The bicarbonate was then added and dissolved, and the solution was cooled and diluted to volume.

The iodate solution used contained in 1 liter

	gm.
Sodium carbonate (anhydrous).....	40.0
Tartaric acid.....	7.5
Potassium iodide.....	10.0
"    iodate.....	0.7
"    oxalate.....	18.4

In the three methods last described the excess iodine was titrated with 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> with a starch paste indicator made by boiling puffed rice with water. Sucrose was hydrolyzed as described above, but in a volume of 10 cc. with 1 drop of the enzyme preparation.

With the bicarbonate solution the determination was made as follows:

The sugar solution was made to a final volume of 10 cc. in a 50 cc. centrifuge tube. 5 cc. each of Parts A and B of the oxidizing solution were added. The tube was placed in the boiling water bath and covered with an inverted 100 cc. beaker. After heating 15 minutes, the tube was cooled in running water. It was then centrifuged in an International size 1, type S.B. centrifuge at 1800 R.P.M. (8 on the rheostat) for 5 minutes. The supernatant liquid was poured off and the Cu<sub>2</sub>O was washed twice with 10 cc. portions of water, with centrifuging at the same rate and for the same time as above. 10 cc. of the iodate solution were pipetted into the tube, followed by 10 cc. of N H<sub>2</sub>SO<sub>4</sub>, and the Cu<sub>2</sub>O was dissolved completely with the aid of a stirring rod. The titration may be made in the centrifuge tube, but it was found more convenient to transfer the mixture to a small Erlenmeyer flask. The excess iodine was absorbed by 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, the starch paste indicator being used. The difference between this titration and that obtained from a blank carried through with water instead of the sugar solution gave the thiosulfate equivalent of the copper reduced.

All the reagents were checked with pure sugars with the following results.

*Fehling's Solution*—With amounts of glucose less than 5 mg., results somewhat parallel to those of Peters (6) were obtained with the first preparation of Fehling's solution. Later results with solutions prepared from other high grade samples of Rochelle salts were not in agreement with the first series. Fehling's solution, as used in this study, cannot be relied on for the determination of amounts of glucose below 5 mg. In the presence of 5 mg. or more of sugar the differences observed with different tartrates were negligible.

From 5 to 25 mg. of glucose (the largest amount used) the results agreed satisfactorily with those of Quisumbing and Thomas. With sucrose, however, regular differences were obtained. The relation between copper and invert sugar from 5 to 25 mg. of sugar is expressed by the equation

$$y = 0.482x + 0.000464x^2$$

in which  $y$  = mg. of invert sugar and  $x$  = mg. of copper. Since Quisumbing and Thomas used mixtures of equal parts of glucose and fructose instead of hydrolyzed sucrose, the difference noted may be due to the effect of the enzyme preparation, although in the amounts used it gave no blank with the reagents. The difference decreases as the amount of sugar increases and nearly disappears at 25 mg.

*Shaffer-Hartmann Reagent*—The results for glucose presented by these authors ((2) p. 381, Table III, Part 2) were confirmed and it was found that invert sugar gives results identical with those for glucose.

*Tompsett's Reagent*—Perhaps because of the separation of the two parts of the reagent, the results were not in exact agreement with those of Tompsett. They are presented in Table I.

Over most of the range the relationships are linear but breaks occur at low concentrations of the sugars so that linear equations cannot be used to cover the entire range.

*Bicarbonate Solution*—From 0.2 to 3.5 mg. of the sugars, the relations between sugars and copper obtained with this reagent are expressed by the equations

$$\begin{aligned} \text{For glucose } y &= 0.048 + 0.338x \\ \text{“ sucrose } y &= 0.052 + 0.313x \end{aligned}$$

in which  $y$  = mg. of sugars and  $x$  = mg. of copper.

The bicarbonate reagent is preferred to that of Tompsett for the following reasons. (1) The ratio of sugar to copper is lower. (2) This relationship is linear throughout the range used. (3) The cuprous oxide is somewhat more readily compacted in the centrifuge.

*Experiments with Plant Extracts*—Use was made of the 80 per cent alcoholic extracts of various plant materials that were available in the laboratory as follows:

Sample 1.	Leaves of young string bean plants
“ 2.	Stems “ “ “ “ “
“ 3.	Leaves and stems of tomato “
“ 4.	Fruit spurs from Baldwin apple trees
“ 5.	Radish leaves
“ 6.	“ roots

Suitable aliquots of these extracts were freed of alcohol. The water suspensions were cleared with neutral lead acetate and diluted to 500 cc. After filtering, the excess lead was removed by  $\text{Na}_2\text{C}_2\text{O}_4$ . The lead oxalate was filtered off and the cleared extracts were preserved with toluene. Of all these cleared extracts except Sample 5, 25 cc. contained enough sugar to give reliable results with Fehling's solution and 5 cc. contained amounts that could be determined satisfactorily by the other methods. Portions of 10 cc. each of Sample 5 were used with the carbonate reagents. The results obtained, expressed as per cent of the fresh weight of the original plant material, are presented in Table II. As there is no absolute method for determining the amounts of sugars in plant extracts, the appraisal of these results must be somewhat arbitrary.

TABLE I  
*Sugar Equivalent to Copper Reduced. Tompsett's Reagent*

Cu	Glucose	Sucrose
mg.	mg.	mg.
0.5	0.29	0.25
1.0	0.54	0.49
2.0	1.00	1.02
3.0	1.42	1.41
4.0	1.84	1.80
5.0	2.26	2.20
6.0	2.66	2.61
7.0	3.07	3.01
8.0	3.48	3.41
9.0	3.90	3.82

It is probably safe to assume that the reagent giving the lowest amount of sugar in a given extract has determined all the true sugars present, and that the excessive values obtained by the other methods are due to non-sugars to which these reagents are sensitive. It follows that no one method is best for all plant materials.

A possible exception may be noted in Sample 5 (radish leaves). Perfect blanks with Fehling's solution were obtained with 25 cc. aliquots of the cleared extract. However, this may occur in the presence of slightly more than 1 mg. of pure sugar. For this reason the results obtained by the bicarbonate reagent are not necessarily excessive by more than 0.02 to 0.03 per cent.

The Shaffer-Hartmann method cannot be used with extracts of fruit spurs (Sample 4). The values for reducing sugars are very high and so erratic that no attempt was made to calculate sucrose.

TABLE II  
*Sugars in Plant Extracts in Per Cent of Fresh Weight*

Sample No.	Direct reducing as glucose determined by				Sucrose determined by			
	Fehling's solution	Bicarbonate solution	Tompsett reagent	Shaffer-Hartmann reagent	Fehling's solution	Bicarbonate solution	Tompsett reagent	Shaffer-Hartmann reagent
1	0.48	0.47	0.52	0.50	0.35	0.36	0.32	0.32
2	0.58	0.65	0.69	0.69	0.14	0.13	0.13	0.12
3	0.78	0.73	0.76	0.80	0.58	0.62	0.60	0.54
4	0.56	0.57	0.72	1.01	0.26	0.23	0.20	
				1.12				
5	0.00	0.10	0.12	0.12	0.00	0.06	0.04	0.05
6	1.20	2.57	2.60	2.61	1.04	0.71	0.62	0.65
7*	2.11	1.86		2.91				

\* Sample 7 is an extract of apple leaves. The analysis was made by N. W. Stuart.

TABLE III  
*Recovery of Added Sugars*

Solution No.	Bicarbonate solution		Tompsett reagent		Shaffer-Hartmann reagent	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
1	1.01	1.00	1.01	1.04	0.95	1.03
2	1.01	1.02	1.03	0.99	0.99	1.00
3	0.99	0.99	1.06	0.93	0.97	1.01
4	0.95	1.00	1.02	1.00		
5	1.03	1.02	1.02	0.93		
6	0.97	0.99	0.97	0.99		

*Recovery of Sugars Added to Plant Extracts*—Of each of the six cleared plant extracts, 50 cc. were pipetted into a 100 cc. volumetric flask. Solutions were added containing 20 mg. each of glucose and sucrose. After diluting to volume and mixing, the solutions were preserved with toluene. Direct reducing sugars and total sugars after hydrolysis with invertase were determined

in 5 cc. aliquots of these solutions. These aliquots contained one-half the sugars present in similar portions of the original cleared extracts and, in addition, 1 mg. each of glucose and sucrose. Determinations by the Shaffer-Hartmann method were made only on Solutions 1, 2, and 3. The results obtained are listed in Table III. The results for sucrose determined by the bicarbonate reagent were calculated as follows: The copper obtained by direct reduction was subtracted from the total copper. The difference was calculated as sucrose by the equation given above.

Since the relationships between sugars and copper obtained by this reagent are linear throughout, this method is logical, and actually gave excellent results. Applied to the determinations by the other reagents, however, it was far from satisfactory.

TABLE IV

*Fehling's Solution. Recovery of Sucrose in Presence of 5 to 15 Mg. of Glucose*

Sucrose added mg.	No. of determinations	Average sucrose recovered mg.
1	5	1.11
1.55	6	1.24
2	12	1.97
3	12	3.01
5	14	5.02
10	10	9.99

This is to be expected from the fact that the relationships between sugars and copper are not linear throughout the entire range. For this reason two somewhat empirical methods were tried, as follows: (1) The total copper was calculated as invert sugar, direct reducing sugar in the aliquot (as glucose) was subtracted, and the difference multiplied by 0.95. (2) The total copper was calculated as sucrose, direct reducing sugar in the aliquot (as glucose) was subtracted, and the difference considered sucrose.

The second method gave excellent results with all the reagents except the bicarbonate. That this method is satisfactory for Fehling's solution is shown by Table IV which was calculated from the reducing power of mixtures of the pure sugars. From these results it is evident that as little as 2 mg. of sucrose may be determined by Fehling's solution with reasonable accuracy in the

presence of 5 mg. or more of glucose. Smaller amounts of sucrose give erratic results.

#### SUMMARY

No one of the four oxidizing reagents is best for all the plant extracts used.

As far as the materials studied are indicative, it appears that a choice may be made by comparing Fehling's solution and the bicarbonate reagent.

Comparison with the Shaffer-Hartmann reagent is suggested, as because of its convenience it is preferred for use with extracts to which it can be applied.

As used in this study, Fehling's solution is not reliable for the determination of amounts of glucose less than 5 mg. It may be used to determine as little as 2 mg. of sucrose in the presence of 5 mg. or more of glucose.

The calculations of sucrose determined in the presence of reducing sugars must be made in a manner suited to the peculiarities of the oxidizing reagent used.

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# THE EFFECT OF ADRENALIN UPON THE FREE MUSCLE SUGAR AND TOTAL CARBOHYDRATE

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## INTRODUCTION

A possible explanation for the observation (1) that the muscle sugar of adrenalized rabbits sacrificed in the recovery period may be low as compared with controls is that some carbohydrate reserve other than glycogen is depleted during adrenalin activity. Indirect evidence to support this hypothesis has been presented (2). In the present studies the problem was attacked by following the effect of adrenalin upon both the glycogen and the total carbohydrate of the muscle. The difference between the total carbohydrate and glycogen plus muscle sugar gives a new carbohydrate fraction, a reserve of unknown constitution, sometimes alluded to as the bound carbohydrate.

Since it was rather important to determine whether or not a fall in muscle sugar following adrenalin could be demonstrated in animals other than the rabbit, a series of muscle sugar determinations was included for the rat.

## Procedure

Rabbits which had been fasted 24 hours were used and litter mates were selected as controls. 0.5 mg. per kilo of adrenalin was given subcutaneously and the animals were sacrificed under amytal anesthesia 3, 8, and 24 hours after dosing. The animals were given amytal until the eye reflex was gone. The sciatic nerve was severed before removal of the tissue. Analyses for the glycogen, free muscle sugar, and total carbohydrate of the leg muscle of each animal were made, the fermentable fraction of the total reducing power being determined. The muscles of the thigh were used collectively for the carbohydrate analyses, one side serving

for muscle sugar determination, the other for glycogen and total carbohydrate. The skinned sacrolumbalis was taken for fat analysis. Motram's method was used for the liver and muscle fat analyses, and Pflüger's for the glycogen determinations. Urine sugar and nitrogen were determined for the period studied.

The rats were sacrificed by spinal transection according to the method of Anderson and Macleod (3).

#### *Determination of Carbohydrate*

*Fermentable Muscle Sugar*—The procedure of extracting the muscle with boiling water was described in a previous paper (1). To 60 cc. of the filtrate, 15 cc. of 10 per cent  $ZnSO_4 \cdot 7H_2O$ , and 15 cc. of 5 per cent NaOH were added (Somogyi's blood method). 40 cc. of this filtrate were pipetted into a 50 cc. volumetric flask. The pH was adjusted to approximately 5.0 and the contents made to volume. The total amount was shaken 2 minutes with 4.5 gm. of Lloyd's reagent, filtered, and the sugar determined by the Shaffer-Hartmann method. A 15 cc. centrifuge tube, which contained 0.5 gm. of yeast previously washed three times, was filled with the filtrate which was allowed to ferment  $\frac{1}{2}$  hour. The contents of the tubes were again centrifuged and the sugar determined in the same manner as before. Glucose added to filtrates either before or after fermentation was quantitatively recovered.

This procedure yields a filtrate free from hexosephosphate, since the reduction values for the fermentable sugar after 3 hours acid hydrolysis in 2.2 per cent HCl were the same as those before hydrolysis (six determinations). In these experiments the filtrate after hydrolysis was subjected to the same precipitating agents as before hydrolysis, since creatine, which is not originally completely removed, is converted to creatinine during hydrolysis. Moreover, attempts to recover hexosephosphate from the protein-free filtrate by precipitation from ammoniacal alcoholic solution as the magnesium salt failed to yield more than 4 mg. per 100 gm. of tissue of glucose equivalent by the Shaffer-Hartmann reduction.

*Fermentable Total Carbohydrate*—Muscle was quickly excised from the animal which had been given amytal, and frozen with  $CO_2$  ice.<sup>1</sup> 25 gm. of muscle shavings were placed into a 500 cc.

<sup>1</sup> In the first attempts to determine total carbohydrate, the muscle was excised as quickly as possible, weighed, and plunged into the boiling acid.

flask containing 160 cc. of boiling 2.2 per cent HCl and refluxed for 3 hours, or first placed into cold HCl and allowed to stand  $\frac{1}{2}$  hour to allow the acid to penetrate. No significant difference was obtained when the latter procedure was compared with the method of initial boiling. After boiling, the contents of the flask were cooled, neutralized, and made to a volume of 200 cc. From this point the procedure was the same as that described for the determination of fermentable muscle sugar. It is essential that the same volume of filtrate before and after fermentation be used in the Shaffer-Hartmann reduction, since the effect of concentration upon the reduction value does not run parallel for the fermentable and non-fermentable fractions. It was found necessary to add glucose after fermentation and to determine the per cent of recovery. The average for a large series of determinations was 94 per cent. In every instance the individual correction was applied. Ochoa (4), using the Shaffer-Hartmann method for the determination of total carbohydrate on filtrates deproteinized with mercury, also found an inhibiting effect of the filtrate upon copper reduction. Ronzoni *et al.* (5), however, reported quantitative recovery.

It is conceivable that both reducing and fermentable non-carbohydrate substances might be present in the muscle extracts and, therefore, questionable whether the difference between the total fermentable reducing power expressed as glucose equivalent and glycogen and muscle sugar could be regarded as carbohydrate without reservation. A comparison of the fermentable reducing fraction was made with the reducing power of the copper-lime precipitate. The copper-lime precipitate was dissolved in dilute  $H_2SO_4$ ; copper was removed with  $H_2S$ . The effect of fermentation was also determined in this fraction and finally carbohydrate was determined in all fractions by the indole colorimetric method of Dische and Popper.<sup>2</sup>

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No attempt was made to shave the sample. In three out of five determinations so made, the glycogen analysis was higher (by 238, 190, and 137 mg. per 100 gm., respectively) than the total carbohydrate determination, indicating that a considerable amount of carbohydrate did not reach the glucose stage.

<sup>2</sup> For the method see Dische, Z., and Popper, H., *Biochem. Z.*, **175**, 371 (1926).

An example of the results obtained with an acid hydrolysate of rabbit muscle is given.

	Glucose equiva- lents in mg. per 100 gm.
Fermentable reducing fraction in original filtrate.....	456
"      substances by indole colorimetric method.....	480
"      reducing fraction recovered from copper-lime precipitate.....	408
Glycogen in original muscle.....	150

Evidence is thereby presented that a considerable portion of the so called bound carbohydrate fractions represent carbohydrate. The error involved in the quantitative estimation is, however, considerable.

*Non-Fermentable Carbohydrate*—The filtrates for the total carbohydrate determination of both control and adrenalinized animals were tested for carbohydrate after fermentation by the modified Molisch test as described by Foulger (6). A positive test was obtained which was compared colorimetrically with known glucose standards. No significant difference for control and dosed rabbits was noted, the values as glucose equivalents ranging from 12 to 25 mg. per 100 gm., which is less than 5 per cent of the total carbohydrate in normal muscle. The possible significance of the error introduced by the non-fermentable or slowly fermentable carbohydrate was therefore eliminated.

### Results

*Fermentable Muscle Sugar*—The values for fermentable muscle sugar determined for rabbits are given in Table I. The differences between controls and values of adrenalinized animals range between 4 and 6 times the standard deviation of the mean. Only in one instance did the value for the adrenalinized animal fall within the lower limits of normal of the control series. Two determinations of fermentable muscle sugar were made 3 hours after the administration of adrenalin. These values are considerably above the basal range. The earlier work in which the correction for the non-fermentable fraction was not applied and in which sampling was performed after stunning is confirmed at least qualitatively. In the recovery period following adrenalin adminis-

TABLE I

*Effect of Adrenalin upon Fermentable Muscle Sugar, Fermentable Total Carbohydrate, Glycogen, and Fat*

	Muscle sugar	Muscle total carbo- hydrate	Muscle glycogen	Muscle fat	Liver fat
	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	gm. per 100 gm.	gm. per 100 gm.
Control rabbits fasted 32 hrs.	35 38 35 40 35 26 36	522 628 409 450 518 563 610	409 371 295 350 364 333 —	0.54 0.93 — 0.72 1.3 0.85 0.65	2.3 4.2 — 4.7 4.3 5.2 5.3
	35 ± 1.7	529 ± 30	354 ± 16	0.83 ± 0.12	4.3 ± 0.4
Rabbits dosed with 0.5 mg. adrenalin per kilo and killed 8 hrs. later	18 5 17 19 26	46 58 100 74 100	51 20 78 45 45	0.73 0.59 — 0.89 0.85	2.6 3.3 3.4 3.6 4.5
	17 ± 3.3	76 ± 11	48 ± 9	0.77 ± 0.07	3.4 ± 0.3
Rabbits dosed with 0.5 mg. adrenalin per kilo and killed 3 hrs. later	136 172	235 300	75		
Control rabbits fasted 48 hrs.	31 27 37 28 45	423 565 550 575 532	323 250 357 302 227	0.57 — — 0.32 1.07	4.0 — — 3.8 3.4
	34 ± 3.3	529 ± 28	292 ± 23	0.69 ± 0.16	3.6 ± 0.2
Rabbits dosed with 0.5 mg. adrenalin per kilo and killed 24 hrs. later	25 14 14 17 13	104 172 90 166 166	28 44 63 127 58	0.60 — — 0.43 1.40	4.9 — — 1.8 4.3
	17 ± 2.1	140 ± 17	64 ± 16	0.74 ± 0.22	4.1 ± 0.8

All carbohydrate values are given as glucose equivalents.

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tration the muscle sugar falls below the normal range. Quantitatively there was observed a greater difference between values of control and dosed animals when the samples were taken after stunning than when they were taken under amyta<sub>l</sub> anesthesia. This difference cannot be relegated to the non-fermentable fraction, which remains relatively constant. Five determinations of the non-fermentable fraction made after stunning showed no significant difference when compared with the determinations made under amyta<sub>l</sub>. The fermentable fraction of controls killed by stunning ranged from 44 to 60. The mean fall in muscle sugar

TABLE II  
*Effect of Adrenalin upon Fermentable Muscle Sugar of Rat*

Treatment of animals	Individual values	Mean + standard deviation of mean.
	<i>mg. per 100 gm.</i>	
Control rats fasted 27-48 hrs.....	21, 35, 18, 26, 29, 33, 21, 16	25 ± 2.5
Adrenalinized rats. 0.2 mg. per kilo. Sacrificed by spinal transection 3-4 hrs. after dosing.....	32, 34, 23, 8, 44, 27	28 ± 4.9
Adrenalinized rats 1.0 mg. per kilo. Sacrificed by spinal transection 6 hrs. after dosing.....	45, 52, 42	
Adrenalinized rats. 1.0 mg. per kilo. Sacrificed by spinal transection 8 hrs. after dosing.....	20. 2. 15. 0. 20. 28. 20. 21. 15	16 ± 3.0

during the recovery period as determined in stunned animals (total eleven) was 45. These data were compiled from our earlier work. Sampled under amyta<sub>l</sub>, the difference is 19.<sup>3</sup>

The difference between the mean fermentable muscle sugar values for the control rats and that for the rats which received

<sup>3</sup> A young control rabbit and a litter mate, which had received 0.5 mg. of adrenalin subcutaneously per kilo, were killed by spinal transection the 8th hour after dosing. The fermentable muscle sugar of the control rabbit was 33 mg. per 100 gm., that of the adrenalinized animal, 14. These values are characteristic of those obtained under amyta<sub>l</sub> anesthesia.

0.02 mg. of adrenalin per 100 gm. is not significant. It should be noted, however, that while the spinal transection was successful for all the controls, three of the adrenalinized animals showed some twitching in the lower limbs following transection. Since the values of the twitching muscles were no higher than the control range, and two of the satisfactory determinations for dosed animals were as high as the control range, a larger series of determinations was not made. With the exception of one low value for the adrenalinized animals, there was no evidence of a lowering of muscle sugar in the rat following the administration of 0.02 mg. of adrenalin per 100 gm. See Table II. Three of the animals which received 0.10 mg. of adrenalin per 100 gm. were sacrificed 6 hours after dosing. The muscle sugar values obtained are higher than those of the control group. This was to be expected as the blood sugars at this period were over 200 mg. per 100 cc. At 8 hours, when the blood sugars had returned to the resting level, the muscle sugar values ( $16 \pm 3.0$ ) averaged significantly below the control values ( $25 \pm 2.5$ ). It should be noted that two of the values are vanishingly low, while the remaining values are within the range of the lower limits of the control group.

The data (Tables I and II) show that a low muscle sugar value is more readily produced in the rabbit than in the rat. The nature of this species difference is obscure. It is possible that it is in some way tied up with the carbohydrate reserve other than glycogen. The changes in the total carbohydrate of the rat remain to be investigated.

In view of the known errors in the available muscle sugar methods it cannot be positively held that the low muscle sugar values obtained after adrenalin prove that muscle sugar is being rapidly converted to some other carbohydrate form in this period. An alternate explanation would be that in adrenalinized animals there is no postmortem liberation of glucose, a process taking place in normal animal tissues. We feel that the bulk of evidence favors our original explanation (2).

#### *Glycogen*

The mean muscle glycogen content, as recorded in earlier work, of eleven normal rabbits fasted 24 to 29 hours (determined after stunning), was  $282 \pm 28$ . In the present studies, when the muscle

was excised under amytal anesthesia, the mean for nine rabbits was  $326 \pm 21$ . According to the studies of Anderson and Macleod (3), amytal gives lower values in the rat than killing by spinal transection. At any rate the values obtained by us are higher than when the animal was stunned, but the differences are not great. Greater differences were expected since the method of stunning is now generally regarded as producing prodigious loss in glycogen. The glycogen values for controls fasted 48 hours were only slightly lower than those for controls fasted 24 to 32 hours, the mean being  $293 \pm 23$ . Sahyun and Luck (7) found a much greater difference, 497 against 166.

#### *Total Carbohydrate*

In six instances the determination of total carbohydrate was made for two corresponding muscles of the same animal. Differences ranging from 18 to 55 mg. per 100 gm. were obtained. For control animals the variation was 10, 10, 8, 5, 6, and 5 per cent, respectively. In working with frogs, Kerly (8) found a 10 per cent variation for the total carbohydrate content of two corresponding muscles. The individual variation for the glycogen content of different muscles of the rabbit was not determined in the present studies, since the experience of other workers indicated that a variation of 10 per cent was not exceeded when a composite analysis of a group of muscles was made (9-11).

In the control series, whether for rabbits fasted 32 or 48 hours, the fermentable total carbohydrate value in all cases materially exceeded the sum of glycogen and muscle sugar. Kerly found even larger differences for frog muscle. The individual variation in both her and in our experiments is large and is undoubtedly influenced by the errors of sampling. The mean difference between total carbohydrate and glycogen plus muscle sugar is 140 mg. per 100 gm. for our controls fasted 32 hours, and 203 for the controls fasted 48 hours. These values do not include the non-fermentable carbohydrate estimated by the Molisch test nor the small amount of glycogen known not to be hydrolyzed in the determination of the total carbohydrate. The true difference is greater by 50 mg. per 100 gm. at least than the values given. The picture after adrenalin is entirely different. 8 hours after dosing, the mean difference between total carbohydrate and muscle sugar

plus glycogen is only 11. 24 hours after dosing, the difference is 59. The value (total carbohydrate)-(glycogen + muscle sugar) exceeds 3 times the standard deviation of the mean when a comparison of this value for controls and adrenalinized rabbits is made. Excellent evidence is, therefore, presented that adrenalin not only depletes the glycogen reserves but the other reserve of unknown chemical constitution which at present can only be approximated. The results are given in Table I.

TABLE III\*  
*Carbohydrate Balance Sheet of 2 Kilo Rabbit 8 Hours after Subcutaneous  
 Injection of 0.5 Mg. of Adrenalin per Kilo*

	Control	Dosed	Calculation	
	mg.	mg.	10	× mg. per cent
Muscle sugar.....	360 ± 20 (9)†	160 ± 26 (7)	2	×
Blood " .....	200	200	2	" "
Muscle glycogen .	3260 ± 210 (9)	510 ± 110 (6)	10	" "
Bound carbohy- - drate.....	1400 ± 300 (6)	110 ± 110 (5)	10	" "
Urine sugar.....		1700 ± 270 (10)		
Liver glycogen...	323 ± 58 (8)	816 ± 152 (5)	0.53	×
	5543 ± 370	3496 ± 345		

64 per cent of the carbohydrate was accounted for by mobilization.

\* In striking the balance sheet, two alternative methods were possible: to use only data of experiments in which complete analyses for all constituents were made or to include all available data which were gathered under strictly standardized procedures. The latter method was used and will explain why the figures in Tables I and III are not exactly the same. It should also be noted that total carbohydrate could have been used instead of the sum of the three individual components determined. In that case the standard deviation of the mean would have been considerably less.

† The figures in parentheses indicate the number of determinations made.

#### DISCUSSION

In the present studies, the mean variation for the muscle sugar, muscle glycogen, bound carbohydrate, and urine sugar, for both controls and animals dosed with adrenalin is sufficiently small so that reasonable accuracy is assured in striking a carbohydrate balance sheet. Since lactic acid has returned to basal values during the recovery period and since no appreciable amount of

lactic acid is lost in the urine, it may be omitted in a balance. Hexosephosphate obviously is included and makes up a considerable portion of the bound carbohydrate fraction. The calculations given in the balance sheet in Table III are based on the assumption that the peripheral tissues constitute 50 per cent of the body weight. The average weight of the liver of a 2 kilo rabbit was 53 gm. The results show that only 51 per cent of the carbohydrate is accounted for by considering muscle and blood sugar, muscle glycogen, bound carbohydrate, and urine sugar. No data were obtained for liver glycogen in the present studies, but if Sahyun and Luck's and our own data obtained in the past are taken, the increase in the glycogen content of the liver of the adrenalinized animals raises the total carbohydrate accounted for to 64 per cent. *It is important to note* that if the changes in bound carbohydrate were omitted in the present calculations, 83 per cent of the carbohydrate would be accounted for, a figure which would agree almost exactly with that obtained by Cori (12) for the rat, based on analyses, which did not include the bound carbohydrate. The omission of total carbohydrate determinations in earlier experiments might, therefore, appear to invalidate some of the older deductions (11, 13). In the present calculations the difference between the carbohydrate in controls and adrenalinized animals is 4 times the standard deviation of the mean (the square root of the sum of the squares of the individual standard deviations of the mean). Three possibilities are to be considered in accounting for this discrepancy: (1) a decrease in conversion of protein to carbohydrate, (2) the conversion of carbohydrate to fat, and (3) the oxidation of carbohydrate. On a 2 kilo basis, the mean nitrogen excretion for the 8 hour period was  $0.54 \pm 0.04$  gm. for the controls and  $0.54 \pm 0.05$  for the adrenalinized animals. There is, therefore, no indication that the carbohydrate balance would be significantly affected by changes in nitrogen metabolism, a possibility suggested by Watkins and Smith (14).

There is no significant difference between the muscle fat content of adrenalinized and control animals either 8 or 24 hours after the administration of adrenalin. The fat content of the liver is slightly decreased at the 8th hour. It may be concluded that if there is an increased utilization of fat by the peripheral tissues, the fat stores are replenished as fast as they are utilized. Although

the low respiratory quotients observed during adrenalin activity are evidence against the conversion of lactic acid or carbohydrate to fat, there is no evidence from our data that this process might not take place to some extent. The large amount of carbohydrate unaccounted for in the balance sheet would point to such an influence. The hypothesis that fat is converted during adrenalin activity to carbohydrate derives no support from our data.

#### SUMMARY

In rabbit muscle excised under amytal anesthesia, the fermentable sugar 8 and 24 hours after the subcutaneous injection of 0.5 mg. of adrenalin per kilo was significantly lower than that of control litter mates.

In rats killed by spinal transection, the fermentable muscle sugar 3 to 4 hours after the subcutaneous injection of 0.02 mg. of adrenalin per 100 gm. was the same as that of controls. 8 hours after the subcutaneous injection of 0.10 mg. of adrenalin per 100 gm. the fermentable muscle sugar was vanishingly low in certain instances.

The difference between the total fermentable carbohydrate content of the muscle and the sum of the muscle glycogen and free muscle sugar of the rabbit was greatly reduced in the recovery period following adrenalin activity. Evidence is thereby presented that a carbohydrate reserve of unknown constitution as well as glycogen is depleted by adrenalin.

Significant changes in urinary nitrogen excretion or in the fat content of the muscle were not observed following adrenalin activity.

By incorporating total carbohydrate in the balance sheet only 60 per cent of the carbohydrate was accounted for by mobilization.

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## THE RIBOSEPHOSPHORIC ACID FROM XANTHYLIC ACID

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The arrangement of the component parts of the nucleotides derived from ribopolynucleotides is formulated on the basis of the assumed analogy of their structure with that of inosinic acid. The latter nucleotide is the only one from which it had been possible to obtain ribosephosphoric acid by acid hydrolysis and the nucleoside by alkaline hydrolysis. From the other nucleotides only the nucleosides had been isolated; all efforts to obtain a ribosephosphoric acid from them were unsuccessful. The failure was attributed to the greater instability of the ribosephosphoric acid entering in the structure of these nucleotides. The instability, in its turn, was explained by assigning to the phosphoric acid group a position different from that in inosinic acid. The arguments in favor of this assumption have been discussed before and need not be repeated here.<sup>1</sup>

In a recent paper Levene and Dmochowski<sup>2</sup> reported on the fact that xanthyllic acid, derived from guanylic acid, on standing in aqueous solution at its own pH of 1.9 was partly hydrolyzed with the formation of ribosephosphoric acid. The present communication contains a report on the isolation and properties of this acid, and it may now be stated that the part of the theory of the structure of nucleotides which deals with the arrangement of its components is definitely established.

<sup>1</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, **43**, 323 (1920). Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929). Schmidt, G., *Klin. Woch.*, **10**, 165 (1931). Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).

<sup>2</sup> Levene, P. A., and Dmochowski, A., *J. Biol. Chem.*, **93**, 563 (1931).

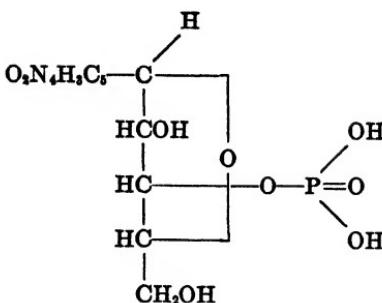
The properties of the ribosephosphoric acid now isolated could be compared with those of the one derived from inosinic acid. The acids were found to differ in several respects. First, they showed different rates of hydrolysis of their phosphoric acid groups, the reaction proceeding twice as fast in the case of the new ribosephosphoric acid. Incidentally, it should be stated that the rates of hydrolysis were compared not directly on the ribosephosphoric acids but on the phosphoribonic acids derived from them (see Table II). This procedure was resorted to for the reason that the phosphoribonic acids were more readily obtained in pure state than were the parent ribosephosphoric acids. Second, differences were observed in the optical changes occurring in course of lactone formation. The changes in rotation observed on the acid derived from inosinic acid were of the same character as those previously reported by Levene and Jacobs<sup>3</sup> and by Levene and Mori,<sup>4</sup> whereas the rotation of the acid obtained from xanthyllic acid remained constant during an equal period of time. Inasmuch as in the ribosephosphoric acid from inosinic acid the inorganic component is attached to the ribose in position (5), it was natural to exclude this position in the case of the ribosephosphoric acid derived from xanthyllic acid. This assumption is substantiated by the fact that this ribosephosphoric acid forms two glycosides, a furanoside and a pyranoside.

All these facts permit of a more accurate formulation of the structure of xanthyllic and hence of guanylic acids. It is now definitely proved that they consist of a ribosephosphoric acid linked glycosidically to the base. Furthermore, the position of the phosphoric acid on the ribose is now restricted to carbon atoms (2) and (3) in view of the facts that position (5) is free and position (4) is engaged in the ring structure, inasmuch as the nucleosides possess the structure of furanosides.<sup>5</sup> Hence, the structure of xanthyllic acid may be represented by the formula:

<sup>3</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 746 (1911).

<sup>4</sup> Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

<sup>5</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931-32).



As has already been stated, the phosphoric acid group may with equal justification be allocated to carbon atom (2). This question is now under investigation.<sup>6</sup>

#### EXPERIMENTAL

*Hydrolysis of Guanylic and Xanthyllic Acids*—The results of Levene and Dmochowski<sup>2</sup> on the hydrolysis of guanylic and xanthyllic acids were confirmed. The estimation of free sugar was performed by the method of Hagedorn and Jensen<sup>7</sup> as modified by Hanes.<sup>8</sup> The phosphorus was determined colorimetrically by the method of Kuttner and Cohn<sup>9</sup> as modified by Raymond and Levene.<sup>10</sup>

In addition, it was found that even at pH 1.5 the rate of hydrolysis of guanylic acid was still very much less than that of xanthyllic acid. Also changing the pH of xanthyllic acid from 1.9 to 1.5 did not greatly affect its rate of hydrolysis. Therefore, xanthyllic acid in an aqueous solution was allowed to stand at its own pH of 1.9, at a constant temperature of 50°, for 3 or 4 days.

*Preparation of Xanthyllic Acid*—Xanthyllic acid was prepared essentially by the procedure of Levene and Dmochowski.<sup>2</sup> It was

<sup>6</sup> In the monograph "Nucleic acids" by Levene and Bass (New York (1931)) there is an error in Fig. VI on p. 190. The phosphoric acid radicle is given there on position (4) instead of position (2) or (3). It is evident from the formula given there that the hydroxyl of carbon atom (4) is engaged in the ring structure.

<sup>7</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

<sup>8</sup> Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>9</sup> Kuttner, T., and Cohn, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

<sup>10</sup> Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).

found that one-third the amount of potassium nitrite used by them was sufficient and possibly better because the excess nitrite seemed to interfere with the precipitation of lead xanthylate. The xanthyllic acid was precipitated from a concentrated aqueous solution by the addition of absolute alcohol. The analysis of this product showed that it was slightly purer than the product obtained by allowing the concentrated aqueous solution to stand in the refrigerator. The xanthyllic acid was not completely precipitated by alcohol but for the preparation of ribosephosphoric acid it was not necessary to isolate it from aqueous solution.

Moist sodium guanylate was used in the preparation of xanthyllic acid. Therefore, the estimation of yields is not very accurate. Approximately 45 to 50 per cent of the theoretical amount of xanthyllic acid was obtained.

*Preparation of Barium Ribosephosphate from Xanthyllic Acid*—1 liter of an aqueous solution containing 10 gm. of xanthyllic acid was allowed to stand in a constant temperature oven at 50° for 3 or 4 days. The solution was then cooled to 20° with tap water, filtered to free it of precipitated base, and treated with a slight excess of a 10 per cent solution of mercuric sulfate. The precipitate, which was sometimes purple in color, was filtered through a Buchner funnel. The solution was neutralized with barium carbonate and saturated with hydrogen sulfide. The excess gas was removed by aeration and the solution treated with barium hydroxide until it was faintly alkaline to phenolphthalein.

This solution was filtered and then concentrated to about 20 cc. under reduced pressure in a bath at about 40°. If any precipitate of barium carbonate separated during distillation, it was removed by filtration. The resulting solution was treated with an equal volume of absolute alcohol, which precipitated the barium ribosephosphate. This was centrifuged, washed twice with absolute alcohol, and then with ether until it became powdery, and was finally dried in a vacuum desiccator over phosphorus pentoxide. The principal impurities were barium phosphate and barium carbonate. (The nitrogen compounds had been completely removed by the treatment with mercuric sulfate.) The yield was 6 gm. or 60 per cent of the theoretical.

*Direct Preparation of Barium Ribosephosphate from Sodium Guanylate*—Approximately 208 gm. of sodium guanylate (from

1600 gm. of yeast nucleic acid) were converted into xanthyllic acid, which was hydrolyzed without isolation. The product was worked up as described above. The yield of barium ribosephosphate was 92 gm. (0.252 mol) or about 49.3 per cent of the sodium guanylate. It had the following composition.

4.108 mg. substance: 21.940 mg. ammonium phosphomolybdate  
0.1000 gm. " : 0.0670 gm. BaSO<sub>4</sub>

C<sub>6</sub>H<sub>10</sub>O<sub>8</sub>PBa. Calculated. P 8.48, Ba 37.59  
Found. " 7.75, " 39.43

*Purification of Barium Ribosephosphate*—Barium ribosephosphate could not be prepared free from nitrogen compounds unless it first underwent the treatment with mercury sulfate. The resulting barium ribosephosphate was purified by two different methods.

*Method I*—The crude product was dissolved in a small amount of water, diluted to 2 or 3 times its volume, and the precipitated barium carbonate and phosphate removed by centrifuging the solution. The clear solution was then reprecipitated by pouring into absolute alcohol. By repeating this process two or three times a satisfactory product was obtained. The following is the analysis of three successive fractions.

1. 6.138 mg. substance: 34.673 mg. ammonium phosphomolybdate (Pregl)

2. 4.201 " " : 22.845 mg. ammonium phosphomolybdate  
3. 3.782 " " : 20.967 " " "

1. 0.1000 gm. substance: 0.0644 gm. BaSO<sub>4</sub>

2. 0.1000 " " : 0.0656 " "

3. 0.1000 " " : 0.0630 " "

C<sub>6</sub>H<sub>10</sub>O<sub>8</sub>PBa. Calculated. P 8.48, Ba 37.59

Found. 1. " 8.20, " 37.89

2. " 7.92, " 38.60

3. " 8.05, " 37.07

*Method II*—The barium was quantitatively removed with sulfuric acid from barium ribosephosphate which was then converted into the dibrucine salt. The solution of brucine salt was filtered and evaporated to a small volume under reduced pressure. The brucine was removed from the precipitated salt by washing with chloroform. The residue was then recrystallized three or four times from 75 per cent acetone-water solution. The analysis of

the main fraction corresponded with that required for dibrucine ribosephosphate. It had the following composition.

4.635 mg. substance: 9.245 mg. ammonium phosphomolybdate

6.1300 " " : 0.301 cc. N (759 mm. and 28°)

$C_{11}H_{18}O_{10}N_4P$ . Calculated. P 3.04, N 5.50

Found. " 2.89, " 5.55

Its rotation was

$$[\alpha]_D^{\infty} = \frac{-0.68^\circ \times 100}{1 \times 2.04} = -33.3^\circ \text{ (in pyridine-water, 1:1 by volume)}$$

The dibrucine ribosephosphate in methyl alcohol was decomposed with barium hydroxide. After removal of methyl alcohol the brucine was removed by filtration and the barium salt precipitated with absolute alcohol. This salt was redissolved in water, made just alkaline with barium hydroxide, centrifuged to remove a precipitate, and reprecipitated with methyl alcohol. The analysis of the dried salt corresponded with that required for barium ribosephosphate.

4.390 mg. substance: 26.460 mg. ammonium phosphomolybdate

0.1000 gm. " : 0.0644 gm.  $BaSO_4$

$C_6H_{10}O_8Pba$ . Calculated. P 8.48, Ba 37.59

Found. " 8.73, " 37.89

*Preparation of Barium Phosphoribonate*—Crude barium ribosephosphate was oxidized with barium hypoiodite following the procedure of Levene and Raymond.<sup>11</sup> After oxidation, the solution of phosphoribonic acid, which had been freed of barium and sulfate ions, was rendered neutral with a methyl alcoholic solution of brucine. On standing in the refrigerator, excess brucine crystallized out and was filtered off. On evaporation under reduced pressure, more brucine separated. Finally, the brucine salt separated and was recrystallized first from methyl alcohol and then from 90 per cent methyl alcohol. Dibrucine phosphate is very soluble in methyl alcohol and would remain in the mother liquors. The analysis of the main fraction corresponded with that required for the tribucine salt of phosphoribonic acid with no water of crystallization.

<sup>11</sup> Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **91**, 751 (1931).

4.385 mg. substance:	7.420 mg. ammonium phosphomolybdate
0.8090 " "	: 0.430 cc. N (769 mm. and 23.5°)
	C <sub>74</sub> H <sub>58</sub> O <sub>21</sub> N <sub>6</sub> P. Calculated. N 5.89, P 2.17
	Found. " 6.17, " 2.45

This brucine salt was converted to the trisodium salt of phosphoribonic acid in the following way. A solution containing 4.56 gm. (0.0032 mol) of the tribrucine salt in methyl alcohol was treated with exactly 0.0096 equivalent of 0.1 N sodium hydroxide. On standing, brucine crystallized out and was filtered off. On evaporation under reduced pressure, further crops of brucine separated and were removed by filtration. The solution was evaporated to a thick syrup, after which it was triturated with methyl alcohol. It became perfectly granular on grinding with fresh portions of methyl alcohol. The salt was dried and analyzed for phosphorus.

4.765 mg. substance:	31.175 mg. ammonium phosphomolybdate
4.516 " "	: 31.040 " " "
.	C <sub>6</sub> H <sub>8</sub> O <sub>9</sub> PNa <sub>3</sub> . Calculated. P 9.93
	Found. " 9.50
	" 9.98

Calcium phosphoribonate (prepared from inosinic acid by Levene and Jacobs<sup>9</sup>) was converted to the sodium salt by shaking with a solution of sodium oxalate. In order to preclude the possibility of the presence of sodium oxalate the calcium salt was added in very small quantities until the solution showed a very slight excess of calcium. The solution of sodium salt was then evaporated and granulated with methyl alcohol as described before. It was analyzed for phosphorus.

4.564 mg. substance:	30.045 mg. ammonium phosphomolybdate
	C <sub>6</sub> H <sub>8</sub> O <sub>9</sub> PNa <sub>3</sub> . Calculated. P 9.93
	Found. " 9.56

*Rotations and Rates of Hydrolysis of Isomeric Phosphoribonic Acids*—The isomeric phosphoribonic acids obtained from the two salts described above were compared as regards their lactonization and the rates of their hydrolysis. The results are given in Tables I and II. In the first and second samples, 0.2574 gm. of dry salt was dissolved in 5 cc. of solution which contained 2.5 cc. (3 equivalents) of 1 N hydrochloric acid. This quantity of salt was

## Ribosephosphoric Acid

taken because it was equivalent to 0.25 gm. of the calcium salt, the rotation of which had been previously reported by Levene and Jacobs<sup>3</sup> and Levene and Mori.<sup>4</sup> In the third sample, 0.2444 gm. of sodium salt was dissolved in 5 cc. of solution containing 1.57 cc. (2 equivalents) of 1 N hydrochloric acid. The readings were taken immediately in a jacketed 1 dm. tube at 7° by Dr. Alexandre

TABLE I  
*Rotations of Phosphoribonic Acids*

Sample 1 From inosinic acid			Sample 2 From xanthyllic acid			Sample 3 From xanthyllic acid. Sodium salt + 2 equivalents of acid		
Time	$\alpha^{25}$	$[\alpha]_{5,461\text{\AA}}^{25}$	Time	$\alpha^{25}$	$[\alpha]_{5,461\text{\AA}}^{25}$	Time	$\alpha^7$	$[\alpha]_{5,461\text{\AA}}^7$
hrs.			hrs.			min.		
0	-0.535	-10.42	0	-0.311	-6.05	0	-0.353	-7.22
1	-0.480	-9.34	0.7	-0.319	-6.20	5	-0.350	-7.15
2.1	-0.436	-8.47	2.1	-0.320	-6.21	15	-0.353	-7.22
4.6	-0.318	-6.18	4.4	-0.325	-6.31	45	-0.353	-7.22
22.4	-0.080	-1.55	22.3	-0.352	-6.85	1097	-0.357	-7.29
			28.5	-0.348	-6.76			

TABLE II  
*Hydrolysis of Phosphoribonic Acids*

From inosinic acid		From xanthyllic acid	
Time	Hydrolysis	Time	Hydrolysis
hrs.	per cent	hrs.	per cent
0	6.35	0	6.85
1	12.6	1	22.5
2	17.0	2	33.0
4	24.8	4	53.1
6	34.1	6	76.1
11.5	46.6	11.5	79.7

Rothen. The light source was the green line, 5.461 Å., of the mercury vapor lamp.

0.5 cc. portions of Samples 1 and 2 in Table I were diluted to 50 cc. in volumetric flasks and the phosphorus determined colorimetrically.<sup>9</sup> These solutions then contained a total of 0.051 mg. of phosphorus per cc. The acid concentration was 0.01 N, which

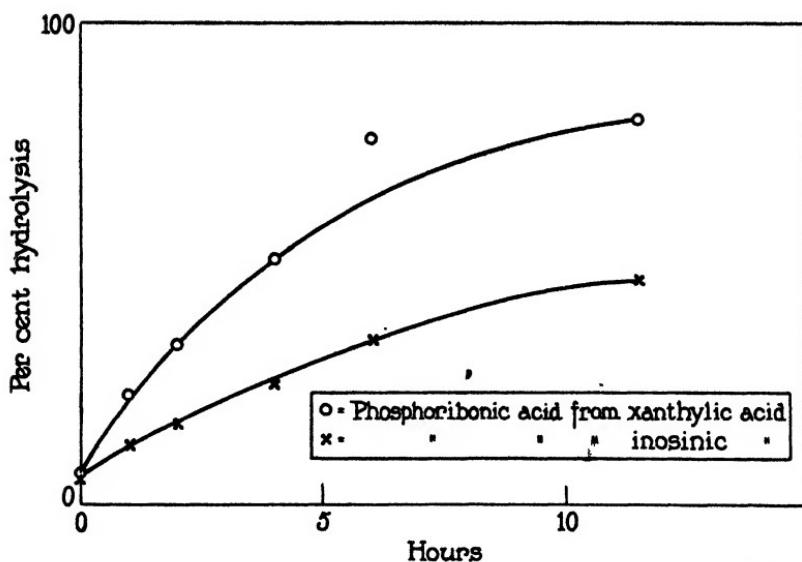


FIG. 1. Rates of phosphate hydrolysis of the phosphoribonic acids

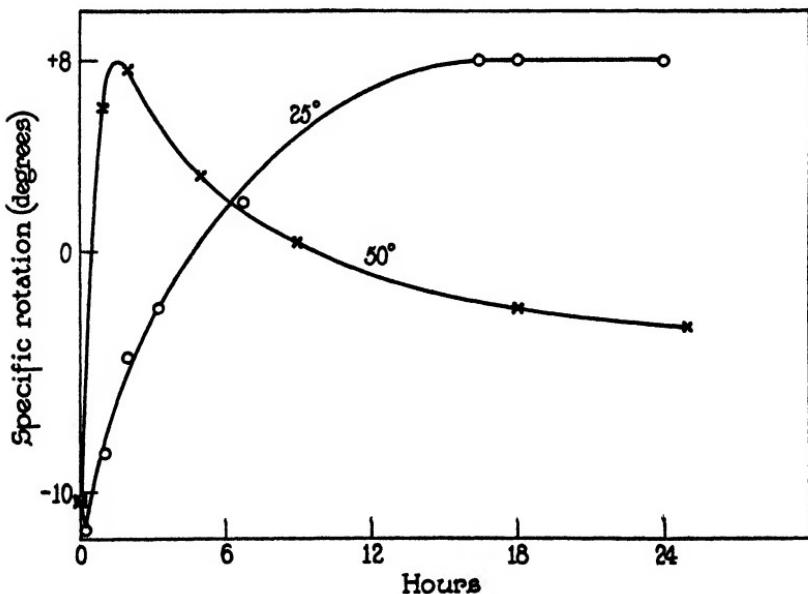


FIG. 2. Glycoside formation of ribosephosphoric acid from xanthyllic acid.

was about equal to that of the free phosphoribonic acid. 5 cc. portions were sealed in test-tubes and hydrolyzed in a boiling water bath for different periods of time. The results<sup>9, 10</sup> are recorded in Table II and Fig. 1. It is concluded from the results of lactone formation and rates of hydrolysis that the phosphoribonic acids, obtained from xanthyllic and inosinic acids respectively, are different.

*Glycosides of Ribosephosphoric Acid*—The glycosides were prepared according to the directions of Levene and Raymond.<sup>12</sup> A solution of ribosephosphoric acid was prepared by dissolving 2.5 gm. of barium ribosephosphate in 100 cc. of methyl alcohol containing 1 gm. of dry hydrogen chloride. The changes in specific rotation of the solutions were observed at 25° and 50°. Fig. 2 shows curves which are typical of the formation of both a <1, 4> and a <1, 5> glycoside. This glycoside formation showed that in the ribosephosphoric acid from xanthyllic acid both the (4) and (5) positions are free.

<sup>12</sup> Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89**, 479 (1930).

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